Copper Deficiency Exacerbates Bile Duct Ligation-Induced Liver Injury and Fibrosis in Rats

Ming Song, Zhanxiang Zhou, Theresa Chen, Jingwen Zhang, and Craig J. McClain

Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition (M.S., J.Z., C.J.M.), Department of Pharmacology and Toxicology (T.C, C.J.M.), University of Louisville Alcohol Research Center (M.S., T.C., C.J.M.), University of Louisville School of Medicine, Louisville, KY 40202; Robley Rex Veterans Affairs Medical Center (C.J.M.), Louisville, KY 40206, USA; and the Department of Nutrition, University of North Carolina at Greensboro, Kannapolis, NC 28081(Z.Z.)
Running Title: Copper and Liver fibrosis

Corresponding author:

Craig J. McClain, M.D.

Departments of Medicine, Pharmacology & Toxicology
Division of Gastroenterology, Hepatology and Nutrition

University of Louisville School of Medicine
505 S. Hancock Street, CTR Rm503
Louisville, KY 40202, USA
Phone: (502)-852-6189
Fax: 502-852-8927
E-mail: cjmccl01@louisville.edu

Number of text pages: 32
Number of tables: 2
Number of figures: 6
Number of references: 40
Number of words: Abstract 231
Introduction 420
Discussion 1023

ABBREVIATIONS: TM, tetrathiomolybdate; BDL, bile duct ligation; NAFLD, non-alcoholic fatty liver disease; Con A, concanavalin A; CCl₄, carbon tetrachloride; SOD₁, copper/zinc superoxide dismutase; SOD₂, manganese superoxide dismutase; ICP-MS, inductively coupled plasma mass spectroscopy; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; H&E, hematoxylin and eosin; α-
SMA, α-smooth muscle actin; CCS, Copper chaperone for SOD1; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; GSH, reduced glutathione; GSSG, oxidized glutathione; Tfb1m, mitochondrial transcription factor B1; SAM, S-adenosylmethionine; mtDNA, mitochondrial DNA; HNE, hydroxynonenal; TGF, transforming growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPARα, peroxisome proliferator activated receptor alpha; Cpt1a, carnitine palmitoyltransferase 1a, liver; Hmgcs2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial); 18SrRNA, 18S ribosomal RNA.

Section: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

Copper levels are elevated in a variety of liver fibrosis conditions. Lowering copper to a certain level protects against fibrosis. However, whether severe copper deficiency is protective against liver fibrosis is not known. The purpose of the present study is to evaluate this question by inducing severe copper deficiency using the copper chelator, tetrathiomolybdate (TM), in a bile duct ligation (BDL) rat model. Male Sprague-Dawley rats were divided into four groups: sham, sham plus TM, BDL, BDL plus TM. TM was given in a daily dose of 10mg/kg BW by means of intragastric gavage, beginning five days after BDL. All the animals were killed two weeks after surgery. Severe copper deficiency was induced by TM overdose in either sham or BDL rats, as shown by decreased plasma ceruloplasmin activity. Liver injury and fibrosis were exacerbated in BDL rats with TM treatment, as illustrated by robustly increased plasma AST and hepatic collagen accumulation. Iron stores, as measured by plasma ferritin, were significantly increased in copper deficient BDL rats. Moreover, hepatic heme oxygenase-1 expression was markedly down-regulated by copper deficiency in BDL rats. In addition, hepatic gene expression involving mitochondrial biogenesis and β-oxidation was significantly up-regulated in BDL rats, and this increase was abolished by copper deficiency. In summary, severe copper deficiency exacerbates BDL-induced liver injury and liver fibrosis, likely due to increased iron overload, and decreased antioxidant defenses, and mitochondrial dysfunction.
Introduction

Copper is an essential trace element for many biological processes, including mitochondrial respiration, iron metabolism, detoxification of free radicals and cross-linking of connective tissue (Bonham et al., 2002). Disruption of copper homeostasis is associated with many human diseases, particularly liver diseases. Excessive copper accumulation in the liver secondary to cholestasis has been well documented in patients with primary biliary cirrhosis (Deering et al., 1977), as well as in alcoholic cirrhotics (Rodríguez-Moreno et al., 1997) and experimental fibrosis (Schaff et al., 1991). Conversely, copper is decreased in the early stage of some liver diseases, such as alcoholic steatosis (Uhlikova et al., 2008), non-alcoholic fatty liver disease (NAFLD) (Aigner et al., 2008 and 2010), and acute experimental liver injury (Domitrović et al., 2008). Therefore, maintaining normal copper homeostasis could be an important therapeutic target for liver diseases.

It is well established that the fibrotic pathway is modulated by copper (Brewer et al., 2004). Anti-copper therapy has been shown to be effective in protecting against bleomycin-induced pulmonary fibrosis (Brewer et al., 2003) and carbon tetrachloride-induced liver cirrhosis in mice (Askari et al., 2004), at least in part, by inhibition of TGF-β. In addition, lowering copper was also demonstrated to protect against both concanavalin A (Con A) (Askari et al., 2004) and acetaminophen (Ma et al., 2004) induced liver injury through inhibition of the inflammatory cytokines, TNF-α and IL-1β. However, early studies showed that severe copper deficiency exacerbated acute carbon tetrachloride (CCL₄)-induced hepatotoxicity (DiSilvestro and Carlson, 1991), whereas
hepatic copper preloading protected against CCl₄-induced liver injury (Barrow and Tanner, 1989). The underlying mechanism(s) remain to be elucidated.

Copper deficiency is associated with decreased antioxidant defenses, including copper/zinc superoxide dismutase (SOD1), cytochrome c oxidase, glutathione peroxidase (Johnson and DeMars, 2004; Prohaska, 1991) and ceruloplasmin. Recently, copper deficiency was observed in NAFLD patients and was correlated with alterations in iron metabolism (Aigner et al., 2008 and 2010). Moreover, dietary fructose interacted with copper deficiency and markedly enhanced the metabolic complications of copper deficiency (Fields et al., 1984), suggesting that copper deficiency might be an important component in the “two-hit” model of nonalcoholic steatohepatitis. The mechanisms for these interactions are not well defined.

Our previous work has shown that lowering copper to 30% of baseline protected against BDL-induced liver injury and fibrosis (Song et al., 2008). However, it is not clear whether or not severe copper deficiency is protective against liver fibrosis. The present study was designed to investigate the effect of severe copper deficiency on the BDL-induced liver fibrosis and explore the potential underlying mechanism(s).
Materials and Methods

Animal Surgery and Experimental Protocol

Seven week old male Sprague-Dawley rats (200-220g) were obtained from the Harlan Laboratories (Indianapolis, IN). Rats were housed in the animal facilities of University of Louisville Research Resources Center on a 12 hour light/dark cycle and fed food and water ad libitum for one week before beginning the experiments. All studies were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. The animals were randomly divided into four groups: sham, sham plus TM, BDL, BDL plus TM. TM[(NH₄)₂MoS₄, PubChem Substance ID 24859366], as an ammonium salt (kindly provided by Dr. George Brewer, University of Michigan, Ann Arbor, Michigan) was dissolved in deionized water. In TM-treated animals, it was given in a daily dose of 10mg/kg BW by means of intragastric gavage, beginning five days after BDL.

Bile duct ligation was performed using a standard technique. Briefly, rats were anesthetized with ketamine and xylazine. After midline laparotomy, the common bile duct was exposed and twice ligated with 1-0 silk suture. Sham operation was performed by gently touching the bile duct. All the animals were killed two weeks after surgery, blood and liver samples were harvested.

Assessment of Copper and Iron Status

Plasma ceruloplasmin was measured on the basis of its oxidase activity (Schosinsky et al., 1974). Copper and iron content in the liver tissue was measured by inductively coupled plasma mass spectroscopy (ICP-MS) after predigestion of the tissues with trace
metal grade nitric acid (Fisher Scientific, Pittsburgh, PA). Plasma ferritin was determined by commercially available kit (ALPCO Diagnostics, Salem, NH).

**Liver Enzyme Assay**

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin assays were performed with commercially available kits (Infinity, Thermo Electron Corp., Melbourne, Australia) based on a colorimetric method.

**Histology and Immunohistochemistry**

Formalin-fixed, paraffin-embedded liver sections were cut at a 3 µm thickness. Liver injury was determined by staining with hematoxylin and eosin (H&E). Extracellular matrix accumulation in liver sections was determined by staining with Masson’s trichrome and Sirius red-fast green. The area in the liver section of positive Sirius red staining was quantified using MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Specifically, a Molecular Devices (Sunnyvale, CA) Image-1/AT image acquisition and analysis system incorporating an Axioskop 50 microscope (Carl Zeiss Inc., Thornwood, NY) was used to capture and analyze eight non-overlapping fields per section at 400×magnification. Data from each section were pooled to determined means. Image analysis was performed using techniques described previously (Bergheim et al., 2006).

For immunohistochemical analysis, sections were incubated with anti-α-smooth muscle actin (α-SMA) (DAKO, Carpenteria, CA) or anti-4-hydroxynonenal (HNE) (Alpha Diagnostic International Inc., San Antonio, TX), for 30 min. 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 tissues using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. For real-time RT-PCR, the first-strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems, Foster City, CA). The reverse transcription was carried out using 1×Taqman RT buffer, 5.5 mM MgCl₂, 500 mM of each dNTP, 2.5 mM random hexamer, 8 U of RNase inhibitor and 25 U of Multiscribe Reverse Transcriptase with 200 ng of total RNA. The RT conditions were 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C. Real-time PCR was performed with an ABI prism 7500 sequence detection system and reactions were prepared using SYBR green master mix (Applied Biosystems, Foster City, CA). Primers were designed and synthesized by SABiosciences (SABiosciences, Frederick, MD) (Genbank Accession Number, see Table 1). The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing with 18Sr RNA gene expression in all the experiments.

**Western Blot**

Hepatic nuclear and cytosol segments were extracted by using commercial available kit (Active Motif, Carlsbad, CA). Equal amounts of protein were loaded and resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). The membrane was blocked and probed with primary antibody for copper chaperone for SOD1 (CCS) (dilution 1:500),
SOD1 (dilution 1:1000), manganese superoxide dismutase (MnSOD, SOD2) (dilution 1:1000), nuclear factor erythroid-2-related factor 2 (Nrf2) (dilution 1:500), heme oxygenase-1 (HO-1) (dilution 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or histone H3 (dilution 1:5000) (Abcam, Cambridge, MA) overnight at 4°C, and then was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein signals were visualized using the enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK). Band intensities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

**Hepatic GSH/GSSG and SAM Assay**

Reduced glutathione (GSH), oxidized glutathione (GSSG) and S-adenosylmethionine (SAM) were determined by HPLC.

**Statistical Analysis**

Results are expressed as mean ± SD (Standard Deviation). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls’ Multiple Comparison Test. $P < 0.05$ was considered statistically significant.
Results

Effects of Copper Deficiency and BDL on Body Weight, Liver Weight, and Blood Metabolites

After 2 weeks BDL, body weight gain was significantly decreased compared to sham operated rats. Both body weight and body weight gain were significantly decreased by TM-induced copper deficiency in BDL rats; however, they were not significantly affected by TM treatment in sham operated rats. Liver weight and liver/body weight ratio were significantly increased after BDL, and they were further increased by copper deficiency. Similarly, plasma cholesterol was significantly higher in BDL rats compared to sham operated rats, and it was further increased by TM-induced copper deficiency, which is a typical sign associated with copper deficiency (Table 2).

Copper and Iron Status

As expected, the plasma ceruloplasmin level was significantly elevated (approximately 2.5 fold) in the rats after two weeks BDL. Conversely, the plasma ceruloplasmin was significantly decreased in both sham and BDL animals treated TM compared to animals with sham operation only (about 8% and 3% of baseline, respectively) (Fig. 1A). Liver copper was significantly increased in BDL rats compared to sham animals, and it was significantly decreased in BDL plus TM rats compared to BDL animals (Fig.1 B). As an indicator of copper status (Harvey and McArdle, 2008), hepatic CCS expression was up-regulated by copper deficiency, and negatively related to the liver copper and plasma ceruloplasmin level, as shown by Western Blot (Fig.1 C). All of the data above suggested that biologically available copper was severely deficient in TM treated animals. The plasma ferritin, a marker of total body iron stores, was robustly
increased (approximately 13 fold) in BDL rats treated with TM compared to controls (Fig.1 D). Liver iron was also significantly increased in both sham and BDL rats treated TM compared to control (Fig.1 E).

Copper Deficiency Exacerbated BDL-Induced Cholestatic Liver Injury

Liver injury was assessed by plasma liver enzymes (ALT, AST, and ALP), total bilirubin, and histology. The plasma ALT, AST and ALP levels were not significantly changed by TM-induced copper deficiency in sham-operated rats compared to controls. After two weeks BDL, plasma ALT, AST and ALP were slightly elevated (differences did not reach statistical significance). However, TM-induced copper deficiency led to a significant increase in plasma ALT level by 2-fold in BDL rats compared with sham-operated animals (Fig.2 A). The plasma AST level was robustly increased by approximately 4-fold and 2-fold respectively in BDL plus TM rats compared to sham and BDL animals (Fig.2 B). Similarly, the plasma ALP and total bilirubin were also significantly elevated in copper deficient BDL rats compared to controls (Fig.2 C and D), suggesting exacerbated cholestatic liver injury caused by copper deficiency in BDL animals.

Bile infarcts are the typical features of cholestatic liver injury due to bile acid induced hepatocyte necrosis. Consistent with the biochemical findings, H&E staining showed extensive bile infarcts, along with distinct neutrophil infiltration in the liver sections of BDL plus TM rats; by contrast, very few bile infarcts were seen in the livers of rats with two weeks BDL alone. Short term copper deficiency induced by TM did not cause significant pathological changes in the livers of sham-operated animals compared with
controls (Fig.2 E). Collectively, these data suggest that copper deficiency exacerbated BDL-induced cholestatic liver injury.

**TM-Induced Copper Deficiency Aggravated Hepatic Fibrosis Induced by BDL**

Hepatic fibrosis was evaluated by Masson’s trichrome and Sirius red staining of liver sections. Collagen content was assessed by morphometrical analysis of Sirius red staining. In sham-operated rat livers, only normal staining around vessels and no fibrosis was observed. TM-induced copper deficiency did not cause overt histological changes in sham-operated rats. After two weeks BDL, collagen accumulation was obvious in both the portal triad and interstitial areas, with bridging fibrosis formation, and this was significantly increased by TM-induced copper deficiency as shown by Masson’s trichrome and Sirius red staining (Fig.3 A and B). Quantification of Sirius red staining by image analysis showed that collagen content was robustly increased (5-fold) in the livers of BDL rats, and it was further significantly increased (8-fold) with TM-induced copper deficiency compared to controls (Fig.3D).

We further evaluated fibrogenesis by immunohistochemistry staining for α-SMA, a marker of hepatic stellate cell activation. Compared with controls, α-SMA expression was markedly increased in the livers of BDL rats, and it was further enhanced by TM-induced copper deficiency, suggesting enhanced fibrogenesis (Fig.3 C). Taken together, our data clearly showed that copper deficiency aggravated BDL-induced liver fibrosis, which paralleled the severity of liver injury.

**TM-Induced Copper Deficiency Inhibited Hepatic Gene Expression Involved in Mitochondrial Biogenesis and Fatty Acid β-Oxidation**
Data from liver enzyme assays suggested that mitochondrial impairment is a likely potential mechanism involved in copper deficiency associated liver injury. To further evaluate mitochondrial function, the gene expression implicated in mitochondria biogenesis and fatty acid β-oxidation was determined by real time RT-PCR (Fig.4). Our data showed that the mRNA expression of the mitochondrial transcription factor B1(Tfb1m), a transcription factor which is necessary for basal transcription of mammalian mitochondrial DNA (mtDNA) (Falkenberg et al., 2002), was significantly up-regulated more than 2-fold in the livers of BDL rats, and this was abrogated in copper deficient BDL rats. The mRNA expression of the key genes regulating fatty acid oxidation, such as, PPAR-α, Cpt1a and Hmgcs2, was significantly increased in BDL rat liver, whereas this increase was blocked in copper deficient BDL rat.

**Increased Oxidative Stress and Decreased Antioxidant Defense by TM-Induced Copper Deficiency in BDL Rats**

To evaluate the possible effects of BDL and copper deficiency on oxidative stress, GSH/GSSG ratio, S-adenosylmethionine (SAM) and 4-HNE were determined. A significant increase in GSH/GSSG ratio (Fig.5 A) was observed, suggesting a response to more oxidative stress in copper deficient BDL rats. Hepatic SAM, a precursor of GSH synthesis, was also significantly decreased in copper deficient BDL rats (Fig.5 B). SODs are important antioxidant defenses against oxidative stress. SOD1, a cuproenzyme, is mainly distributed in the cytosol, and in the inter-membrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001). SOD2 is located in the mitochondria. Hepatic SOD1 expression was significantly down-regulated in both sham and BDL rats in response to TM-induced copper deficiency, as shown by Western Blot (Fig.5 C).
However, hepatic SOD2 expression was not significantly different in all groups (data not show). 4-HNE is the end product of lipid peroxidation, and its adduction to proteins serves as a marker of lipid peroxidation. As shown in Fig.5 D, immunoreactivity of 4-HNE in the liver was increased by BDL, and it was further enhanced by copper deficiency in BDL rats.

**Hepatic Heme Oxygenase-1 (HO-1) Expression was Significantly Inhibited by BDL and Copper Deficiency Interaction**

HO-1 is a stress-related Nrf2 target gene and is an anti-oxidant defense enzyme. As shown in Fig.6A, HO-1 expression was significantly down-regulated by copper deficiency in BDL rat liver. Nrf2 is a transcription factor which regulates the expression of anti-oxidative and other cytoprotective genes, including HO-1. To further understand the molecular mechanism by which copper deficiency and BDL-induced inhibition of anti-oxidant defense, Nrf2 expression was evaluated by Western blot. Hepatic nuclear Nrf2 expression was slightly up-regulated in response to copper deficiency in sham-operated rat liver, whereas it was slightly down-regulated after 2 weeks BDL. However, the differences did not reach statistical significance (data not show).
Discussion

Our previous data have demonstrated that lowering copper to 30% of basal level protected against BDL-induced liver fibrosis (Song et al., 2008). However, when copper is severely deficient (<8% of baseline in terms of ceruloplasmin) (Figure 1A), it exacerbated BDL-induced liver injury and liver fibrosis. Sprague-Dawley (SD) rats are known to be a sensitive model in response to copper deficiency, and low dose TM (10 mg/kg/day) depleted copper to a greater extent in the SD rat than it did in our previous mouse model (Song et al., 2008). It appeared that the severity of liver fibrosis paralleled the severity of liver injury and hepatic stellate cell activation in rats subjected to BDL and severe copper deficiency, suggesting that liver injury is the primary event in the liver fibrosis induced by copper deficiency and BDL. Moreover, liver injury in BDL rats resulting from copper deficiency is characterized by robustly increased plasma AST (Figure 2B), suggesting potential mitochondrial impairment (Panteghini, 1990). In fact, previous work has shown that both acute and chronic severe copper deficiency led to abnormal mitochondria (Gallagher et al., 1973). However, the underlying mechanism(s) are not fully understood.

One of the principal cuproenzymes, cytochrome c oxidase, is the terminal enzyme of mitochondrial respiratory chain. Another cuproenzyme, SOD1, is an important antioxidant defense against oxidative stress. The distribution of SOD1 is mainly in the cytosol, and it has also been found in the inter-membrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001). It is well documented that activity of both cytochrome c oxidase and SOD1 were decreased by copper deficiency (Johnson and DeMars, 2004; Prohaska, 1991). Consistent with previous studies, our data also showed that hepatic...
SOD1 expression was significantly decreased in response to copper deficiency (Fig. 5 C). Increased iron stores secondary to copper deficiency may be another important factor for the mitochondrial damage. Iron export from non-intestinal cells requires ceruloplasmin, the principal copper carrying protein with ferroxidase activity. Ferrous (Fe$^{2+}$) ion must be converted to ferric (Fe$^{3+}$) ion by ferroxidase before being exported out of the cells for transport in the plasma. Ceruloplasmin deficiency prevents iron release from cells and iron accumulates in the liver in macrophages, hepatocytes, and in cells from several other organs (Harris et al., 1999). Our data clearly showed extremely low plasma ceruloplasmin activity in both sham and BDL animals induced by TM (Figure 1A).

Iron plays an essential role in the maintenance of mitochondria, through its two major functional forms: heme and iron-sulfur clusters. Most of the iron within cells is routed to the mitochondria for heme biosynthesis and maturation of Fe-S clusters (Atamna et al., 2002). Heme degradation is controlled predominantly by heme oxygenase (HO). Humans and rodents have two HO isoenzymes, namely HO-1 and HO-2, with HO-1 being the only inducible form in response to oxidative stress (Gozzelino et al., 2010). Mice lacking HO-1 displayed hepatic iron overload and liver injury (Poss and Tonegawa, 1997), whereas induction of HO-1 suppressed inflammation and oxidative stress and protected against steatohepatitis (Yu et al., 2010) and a variety of other types of liver injury (Lin et al., 2010; Yun et al., 2010). In the present study, hepatic HO-1 expression was significantly suppressed by copper deficiency in BDL mice, and this may be another contributor to increased iron stores and liver injury. Moreover, in addition to copper, heme is also an important component of the cytochrome c oxidase subunit (Fontanesi et al., 2008). Thus, it is plausible that disturbing heme homeostasis may also contribute to
decreased cytochrome c oxidase activity. Collectively, copper deficiency associated ceruloplasmin activity inhibition coupled with HO-1 suppression led to iron accumulation in mitochondria, which in turn, led to increased ROS via the Fenton reaction, mitochondria dysfunction, and ultimately, cell death. It appeared that increased iron stores paralleled the severity of liver injury and fibrosis, suggesting it may be an important mechanism underlying exacerbated liver injury by copper deficiency in BDL rats.

Maintaining mitochondrial homeostasis involves biogenesis and replacement. Therefore, we further evaluated mitochondrial biogenesis by determining the related gene expression. We found that the mRNA expression of mitochondrial transcription factor B1(Tfb1m), a transcription factor which is necessary for basal transcription of mammalian mitochondrial DNA (mtDNA) (Falkenberg et al., 2002), was significantly up-regulated more than 2-fold in the livers of BDL rats, and this was abrogated in copper deficient BDL rats. Similarly, the mRNA expression of the key genes regulating fatty acid β-oxidation, such as, PPAR-α (Shalev et al., 1996), Cpt1a (Akkaoui et al., 2009) and Hmgcs2 (Kostiuk et al., 2008) were significantly increased in BDL rat livers, whereas this increase was blocked in copper deficient BDL rats. A recent study showed that mitochondrial biogenesis may be regulated by HO-1 in heart (Piantadosi et al., 2008), which may also explain our results. Taken together, it appears that disruption of mitochondrial biogenesis is another likely mechanism by which copper deficiency exacerbates liver injury in BDL rats.

Previous work showed that the activity of hepatic HO-1 in rats is elevated in response to 5 weeks copper deficiency (Johnson and DeMars, 2004). Our data showed that hepatic
HO-1 expression did not significantly increase in response to copper deficiency in sham-operated rats, probably due to the short term of copper deficiency (10 days). Conversely, HO-1 expression was significantly inhibited in BDL rats in response to copper deficiency, suggesting other factors, such as accumulated bile acids, may play a role. The mechanism responsible for the decreased HO-1 expression is poorly understood. Nrf2 is a transcription factor which regulates the expression of anti-oxidative and other cytoprotective genes, including HO-1 (Yeligar et al., 2010; Yao et al., 2007). However, hepatic nuclear Nrf2 expression in BDL rats was not significantly changed in response to copper deficiency (data not show), suggesting either a disturbance of Nrf2 DNA binding or that other transcription factors might be involved.

In summary, our data demonstrated that copper deficiency may lead to iron overload, possibly, by inhibition of ceruloplasmin activity and HO-1 expression, which in turn, cause mitochondria dysfunction. Severe copper deficiency synchronized with BDL to exacerbate liver injury and fibrosis. Our data provide further insights into the understanding of the role of copper homeostasis in liver injury and fibrosis.
Acknowledgments

We thank Dr. Gavin E. Arteel for the help with image quantification. We thank the support of Center for Regulatory and Environmental Analytical Metabolomics (CREAM) Mass Spectrometry Facility at University of Louisville, and Dr. Richard M. Higashi and Dr. Teresa W.-M. Fan for the help with measuring trace metals.
Authorship Contributions

Participated in research design: Song, McClain.

Conducted experiments: Song, Zhou, Zhang

Performed data analysis: Song, Chen, McClain

Wrote or contributed to the writing of the manuscript: Song, McClain.
References


Footnotes

This study was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grants PO1AA017103, P30AA019360, RO1AA015970, RO1AA018016, RO1AA018869, R37AA010762, RC2AA019385, RO1AA014623, RO1AA016013 (C.J.M.), RO1AA018844 (Z.Z.)]; National Institute of Diabetes and Digestive and Kidney Diseases [Grant RO1DK071765(C.J.M.)]; Louisville VA Medical Center [Grant 5I01BX000350 (C.J.M.)] and National Science Foundation Experimental Program to Stimulate Competitive Research [Grant EPS-0447479].

This manuscript was presented as a poster presentation at the 15th ISHSR (International Society of Hepatic Sinusoidal Research) symposium in Pasadena, California on August 28-September 1, 2010.

Address correspondence to: Craig J. McClain, M.D., Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Louisville School of Medicine,
505 S. Hancock Street, CTR Rm503, Louisville, KY 40202, USA. E-mail: cjmccl01@louisville.edu
Legends for Figures

Fig.1. Effect of TM on copper and iron status 2 weeks after bile duct ligation. Bile duct ligation (BDL) or sham operation (Sham) was performed in male Sprague-Dawley rats as described under Materials and Methods. TM was given in a daily dose of 10mg/kg/day by means of intragastric gavage beginning 5 days after surgery until 2 weeks. Plasma ceruloplasmin (A), liver copper (B), plasma ferritin (D) and liver iron (E) were determined as described in Materials and Methods. Data represent means ± SD (n=5-6). Hepatic Copper chaperone for SOD1 (CCS) expression (C) was examined by Western blot analysis using whole liver cytosol extract, and optical density of band was quantified by Image J software. The ratio to GAPDH was calculated by assigning the value from sham controls as one. Data represent means ± SD (n=3). *, significantly different from sham group. †, significantly different from BDL group. TM, tetrathiomolybdate.

Fig.2. Effect of copper deficiency and BDL on plasma liver enzymes, total bilirubin and liver histology. The animals were subjected to the same treatment protocol as described in Fig. 1. ALT, AST, ALP and total bilirubin (A, B, C, D) were determined in plasma samples by colorimetric assay. Data represent means ± SD (n=5-6). E, representative photomicrographs (100×) of liver sections with hematoxylin and eosin (H&E) staining depicting liver injury as shown by bile infarcts and neutrophil infiltration (arrows). *, significantly different from sham group. †, significantly different from BDL group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.
Fig. 3. Effect of copper deficiency on BDL-induced liver fibrosis. The animals and treatment were the same as described in Fig. 1. Collagen deposition was evaluated by Masson’s Trichrome and Sirius red staining. Hepatic fibrogenesis was assessed by smooth muscle α-actin (α-SMA), a marker of hepatic stellate cell activation. A, representative photomicrographs (100×) of liver sections with Masson’s Trichrome staining. B, representative photomicrographs (100×) of liver sections with Sirius red staining. C, Representative photomicrographs (100×) of immunohistochemistry staining for liver α-SMA. D, quantification of Sirius red positive staining by image analysis. Data represent means ± SD (n=5-6). *, significantly different from sham group. †, significantly different from BDL group.

Fig. 4. Effect of copper deficiency and BDL on the gene expression involving mitochondrial biogenesis and fatty acid β-oxidation. Real time RT-PCR was performed as described under Materials and Methods to determine hepatic Tfb1m, Ppara, Cpt1a, and Hmgcs2 mRNA expression. The expression was normalized as a ratio using 18SrRNA as housekeeping gene. A value of 1 for this ratio was arbitrarily assigned to the data obtained from sham-operated rat. Data represent means ± SD (n=5-6). *, significantly different from sham group. †, significantly different from BDL group. Tfb1m, transcription factor B1, mitochondrial; Ppara, peroxisome proliferator activated receptor alpha; Cpt1a, carnitine palmitoyltransferase 1a, liver; Hmgcs2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial); 18SrRNA, 18S ribosomal RNA.

Fig. 5. Effect of copper deficiency and BDL on hepatic oxidative stress and antioxidant defense system. Hepatic GSH/GSSG ratio and SAM were measured by HPLC (A, B). Data represent means ± SD (n=5-6). Hepatic SOD1 expression (C) was
examined by Western blot analysis using whole liver cytosol extract (except nuclear), and optical density of band was quantified by Image J software. The ratio to β-actin was calculated by assigning the value from sham controls as one. Data represent means ± SD (n=3). D. Representative photomicrographs of the immunohistochemistry staining for 4-HNE in liver section (100×). *, significantly different from sham group. †, significantly different from BDL group. GSH, reduced glutathione; GSSG, oxidized glutathione; SAM, S-adenosylmethionine; HPLC, high-performance liquid chromatography; SOD1, copper/zinc superoxide dismutase; 4-HNE, 4-hydroxynonenal.

**Fig.6. Effect of copper deficiency and BDL on hepatic HO-1 expression.** Hepatic nuclear HO-1 expression was determined by Western Blots. Optical density of band was quantified by ImageJ software. The ratio to GAPDH was calculated by assigning the value from sham controls as one. Data represent means ± SD (n=3). *, significantly different from sham group. †, significantly different from BDL group.
Tables

Table 1

Genbank Accession Number

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfb1m</td>
<td>NM_181474</td>
</tr>
<tr>
<td>Hmgcs2</td>
<td>NM_173094</td>
</tr>
<tr>
<td>Ppara</td>
<td>NM_013196</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>NM_031559</td>
</tr>
<tr>
<td>18SrRNA</td>
<td>X01117</td>
</tr>
</tbody>
</table>

Tfb1m, transcription factor B1, mitochondrial; Hmgcs2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial); Ppara, peroxisome proliferator activated receptor alpha; Cpt1a, carnitine palmitoyltransferase 1a, liver; 18SrRNA, 18S ribosomal RNA.
Table 2

Effects of TM and BDL on Body Weight, Liver Weight, and Plasma Cholesterol

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (BW, g)</td>
<td>284.6 ± 6.0</td>
<td>277.6 ± 14.7</td>
<td>268.5 ± 14.2</td>
<td>237.2 ± 16.9*†</td>
</tr>
<tr>
<td>Body Weight gain(g)</td>
<td>65.20 ± 4.55</td>
<td>52.60 ± 12.26</td>
<td>42.00 ± 15.54*</td>
<td>11.33 ± 13.44*†</td>
</tr>
<tr>
<td>Liver Weight(g)</td>
<td>10.42 ± 0.69</td>
<td>9.46 ± 0.52</td>
<td>12.46 ± 1.49*</td>
<td>15.28 ± 0.77*†</td>
</tr>
<tr>
<td>Liver/BW Ratio(%)</td>
<td>3.659 ± 0.169</td>
<td>3.410 ± 0.139</td>
<td>4.664 ± 0.718*</td>
<td>6.469 ± 0.521*†</td>
</tr>
<tr>
<td>Cholesterol(mg/dl)</td>
<td>94.3 ± 14.2</td>
<td>85.3 ± 4.6</td>
<td>119.6 ± 17.4*</td>
<td>171.7 ± 28.3*†</td>
</tr>
</tbody>
</table>

Seven weeks old male Sprague-Dawley rats were given either BDL or sham operation. TM was given in a daily dose of 10mg/kg/day by means of intragastric gavage beginning 5 days after surgery. All animals were killed 2 weeks after surgery.

Data are shown as means ± SD (n=5-6). *, significantly different from sham group. †, significantly different from BDL group. BDL, bile duct ligation; TM, tetrathiomolybdate.
Figure 1

A

Rat Plasma Ceruloplasmin (mg/dL)

Sham | Sham+TM | BDL | BDL+TM
--- | --- | --- | ---
0 | * | 30 | *
10 | * | 20 | *
20 | | 30 | 
30 | | 40 | 

B

Rat Liver Copper (µg/g wet tissue)

Sham | Sham+TM | BDL | BDL+TM
--- | --- | --- | ---
0 | * | 15 | 
5 | * | 10 | 
10 | | 15 | 
15 | | 20 | 

C

CCS/GAPDH Ratio

Sham | Sham+TM | BDL | BDL+TM
--- | --- | --- | ---
1.00±0.20 | 3.38±0.55* | 1.13±0.22 | 4.01±0.30*†

D

Plasma Ferritin (ng/mL)

Sham | Sham+TM | BDL | BDL+TM
--- | --- | --- | ---
0 | * | 800 | *
200 | | 600 | 
400 | | 400 | 
600 | | 600 | 

E

Rat Liver Iron (µg/g wet tissue)

Sham | Sham+TM | BDL | BDL+TM
--- | --- | --- | ---
0 | * | 120 | *
30 | | 90 | 
60 | | 60 | 
90 | | 90 | 

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 2

A

Plasma ALT (U/L)

Sham | Sham+TM | BDL | BDL+TM

B

Plasma AST (U/L)

Sham | Sham+TM | BDL | BDL+TM

C

Plasma ALP (U/L)

Sham | Sham+TM | BDL | BDL+TM

D

Plasma Total Bilirubin (mg/dL)

Sham | Sham+TM | BDL | BDL+TM

E

H&E

Sham | Sham+TM | BDL | BDL+TM
Figure 3

A
Masson's Trichrome

B
Sirius Red

C
α-SMA

D

Sirius Red Positive Staining (% of Microscope Field)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.25</td>
<td>0.5</td>
<td>0.8</td>
<td>0.75</td>
</tr>
<tr>
<td>Sham+TM</td>
<td>0.25</td>
<td>0.5</td>
<td>0.8</td>
<td>0.75</td>
</tr>
<tr>
<td>BDL</td>
<td>0.15</td>
<td>0.3</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>BDL+TM</td>
<td>0.25</td>
<td>0.5</td>
<td>0.8</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* indicates a significant difference from Sham.
†† indicates a significant difference from Sham+TM.
Figure 4

- **Tph1 mRNA (fold change)**: 
  - Sham 
  - Sham+TM 
  - BDL 
  - BDL+TM

- **PPARα mRNA (fold change)**: 
  - Sham 
  - Sham+TM 
  - BDL 
  - BDL+TM

- **Cpt1a mRNA (fold change)**: 
  - Sham 
  - Sham+TM 
  - BDL 
  - BDL+TM

- **Hmgcr mRNA (fold change)**: 
  - Sham 
  - Sham+TM 
  - BDL 
  - BDL+TM
Figure 5

A

Liver GSH/GSSG Ratio

B

Liver SAM (nmol/g tissue)

C

SOD1

β-actin

SOD1/β-actin Ratio

1.00±0.09

0.59±0.20*

0.85±0.20

0.46±0.09†

D

Sham

4-HNE

Sham+TM

BDL

BDL+TM

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>1.00±0.08</td>
<td>1.16±0.13</td>
<td>0.68±0.43</td>
<td>0.11±0.05*†</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HO-1/GAPDH Ratio