Modest Fructose Beverage Intake Causes Liver Injury and Fat Accumulation in Marginal Copper Deficient Rats

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Objective: Dietary fructose and copper interaction may play an important role in the pathogenesis of nonalcoholic fatty liver disease. In this study, whether or not modest fructose consumption (3% fructose, w/v) (which is more closely related to the American lifestyle with regard to sugar beverage consumption) affects copper status, and causes liver injury and fat accumulation in marginal copper deficient rats was investigated.

Design and Methods: Male weanling Sprague-Dawley rats were fed either an adequate copper (6ppm) or a marginally copper deficient (1.6ppm) diet for 4 weeks. Deionized water or deionized water containing 3% fructose (w/v) was given ad lib.

Results: Modest fructose consumption further impaired copper status in the marginal copper deficient rats and increased hepatic iron accumulation. Liver injury and fat accumulation were significantly induced in the marginal copper deficient rats exposed to fructose.

Conclusions: Our data suggest that modest fructose consumption can impair copper status and lead to hepatic iron overload, which in turn, may lead to liver injury and fatty liver in marginal copper deficient rats. This study provides important information on dietary fructose and copper interaction, suggesting that dietary fructose-induced low copper availability might be an important mechanism underlying fructose-induced fatty liver.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. NAFLD ranges from simple steatosis to steatohepatitis and cirrhosis, is the major cause of abnormal liver enzymes in USA, affecting more than 20% of Americans. Non-alcoholic steatohepatitis (NASH), a more serious form of NAFLD, is present in at least 2-3% of adults (1). The mechanisms underlying NASH are not fully understood. A large part of our knowledge on this disease has been derived from animal studies. Previously used animal models of NASH include genetic models, such as ob/ob or db/db mouse, or nutritional models, such as methionine and choline-deficient diet (2), but these are actually not the real reflections of human disease. The typical characteristics of the Western-style diet include dietary high fat and high fructose corn syrup (HFCS) sweetened beverages (3). The increased consumption of fructose temporally parallels the increased prevalence of obesity and the metabolic syndrome in USA and worldwide (4,5). Emerging evidence has shown that high fructose intake may play an important role in the development of NAFLD (6,7).

The currently used fructose levels in animal studies range from 10% to 30% (w/v) in the drinking water (8-10), or 50-66% of calories derived from dietary fructose (11,12), which are the dietary extremes.
used for mechanistic studies. However, these levels do not reflect the real life style or current nutritional status of Americans. Recently, the American Lifestyle-Induced Obesity Syndrome diet, which is a combination of high fat and HFCS in the drinking water, was introduced to mimic the characteristics of the American fast-food diet (13). The drink used in the American Lifestyle-Induced Obesity Syndrome model is HFCS-55 equivalent (which simulates the 60% of HFCS used in the US food supply), containing 55% fructose and 45% glucose or sucrose at a concentration of 42 g/L, which equals to 2.3-3.3% (w/v) fructose in the drinking water (13,14).

The pathogenesis of NASH likely involves multiple factors. Copper availability is one such factor that was identified very recently (15,16). Both human and animal studies showed that decreased copper availability or inadequate dietary copper intake might play an important role in the pathogenesis of NAFLD. Decreased copper levels were found in a significant proportion of NAFLD patients, and a copper deficient diet induced hepatic steatosis in rats (15,16). Decreased copper availability can be either primary (the average American diet contains only marginal amounts of copper) (17) or secondary. One of the important factors leading to decreased copper availability is high dietary fructose, which has been well documented by Fields and colleagues and by our group (18-21). Moreover, dietary fructose interacts with copper deficiency and markedly enhances the metabolic complications of copper deficiency in rodents, possibly by disrupting copper absorption from gut (18-21). Our group recently showed that high fructose in the drinking water further impaired the copper status of marginal copper deficient rats, and exacerbated liver injury and fat accumulation (21), suggesting that marginal copper deficiency might be an initial component of the “two hit” model of NASH (22). However, the fructose used in the previous studies, including ours (30% fructose in the drinking water), are all high fructose concentration, which is much higher than that consumed by humans. As mentioned above, 3% fructose (w/v) in the drinking water as added sugar sweetener is more relevant to the American lifestyle.

Actually, fructose absorption is saturated at fairly low levels in both human and rat. In rats, the capacity to absorb fructose is 1.5-2.0 g/kg body weight, which is higher than that in humans (0.5 g/kg body weight) (23,24). Thus, the estimated fructose absorption is already saturated even with 3% fructose (w/v). Given that the upper gastrointestinal tract is the common site for the absorption of both copper and fructose (24,25), and the average American diet contains only marginal amounts of copper (17), we hypothesize that even modest fructose (3%, w/v) intake may interfere with copper absorption and impair copper status in marginal copper deficient rats and lead to liver injury and fat accumulation.

Methods and Procedures

Animals
Male weanling Sprague-Dawley rats (35-45 g) from the Harlan Laboratories (Indianapolis, IN) were fed (ad lib) with a purified AIN-76 diet for laboratory rodents with defined copper content. The copper adequate rats received 6 mg/kg copper, and the marginal copper rats were fed with 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 12:12 h light-dark cycle. Animals had free access to either deionized water or deionized water containing 3% fructose (w/v). Fructose enriched drinking water was changed twice a week. All studies were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. At the end of the experiment, all the animals were sacrificed under the anesthesia with pentobarbital at 50 mg/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.

Assessment of copper and iron status
Plasma ceruloplasmin was measured on the basis of its oxidase activity (26). Plasma copper and liver copper were measured by Varian SpectrAA 880/GTA-100 graphite furnace atomic absorption spectrometer (Worcester Polytechnic Institute, Worcester, MA). Liver iron was measured by iCE3000 series flame atomic absorption spectrometer (Thermo Fisher Scientific, Waltham, MA). Plasma ferritin was determined by commercially available kit (ALPCO Diagnostics, Salem, NH).

Hepatic copper, zinc-superoxide dismutase enzyme activity assay
Briefly, liver tissues (less than 9 mg) were homogenized with 10-mM Tris-HCl (PH 7.4). After Mn-superoxide dismutase (SOD) activity was inhibited by 2% sodium dodecyl sulfate (38), SOD activity was measured using SOD determination kit (Sigma-aldrich, St. Louis, MO). The SOD activity was determined by measuring the inhibition rate of formazan dye production.

Liver enzyme and plasma monocyte chemoattractant protein-1 assay
Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercially available kits (Infinity; Thermo Electron Corporation, Melbourne, Australia) based on a colorimetric method. Plasma monocyte chemoattractant protein-1 (MCP-1) was determined by ELISA kit (R&D Systems, Minneapolis, MN).

Glutathione and oxidized glutathione assay
Reduced glutathione (GSH) and oxidized glutathione (GSGG) were determined by HPLC as described previously (27).

Histology and immunohistochemistry
Formalin-fixed, paraffin-embedded liver sections were cut at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Apoptotic hepatocytes were detected by the Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay with an in situ Apoptosis Detection Kit (Millipore, Billerica, MA).

For immunohistochemical analysis, paraffin-embedded liver sections were incubated with 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).
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 (28). Hepatic triglyceride content was determined by commercially available kits (Infinity, Thermo Electron).

Statistical analysis
All data were expressed as mean ± SD and analyzed by 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 \( P < 0.05 \) were considered to be statistically significant.

Results
Effects of low fructose feeding on copper, iron status and hepatic SOD1 activity
Plasma ceruloplasmin activity and plasma copper were significantly decreased in the rats fed with the marginal copper deficient diet, and they were further decreased in marginal copper deficient rats fed with fructose (Figure 1a and b). Similarly, liver copper was significantly decreased in marginal copper deficient rats. However, no significant decrease was observed in those animals additionally fed with fructose (Figure 1c). Hepatic SOD1 activity was markedly decreased in marginal copper deficient rats, as well as in adequate copper rats fed with fructose. However, no further decrease was observed in marginal copper deficient rats additionally fed with fructose (Figure 1d). Liver iron was significantly increased in both adequate copper and marginal copper deficient rats fed with fructose, and it was further increased in marginal copper deficient rats compared to adequate copper rats. However, there was no significant difference in iron levels between adequate copper and marginal copper deficient rats without fructose (Figure 2a). Consistent with the liver iron, plasma ferritin, a marker of total body iron stores, was also significantly increased in marginal copper deficient rats fed with fructose compared to adequate copper rats (Figure 2b).

Dietary marginal copper deficiency and low fructose drinking interact to exacerbate liver injury
Liver injury was assessed by plasma levels of liver enzymes (ALT and AST). Plasma ALT level was not significantly changed by either marginal copper deficiency or fructose feeding. However, marginal copper deficiency and fructose drinking together significantly increased plasma ALT as compared to controls. While there is no significant difference in plasma AST level among the adequate copper rats fed with or without fructose, or in marginally copper deficient rats without fructose, whereas marginal copper deficiency and fructose feeding synergistically increased plasma AST level compared to the other groups (Figure 3a). Consistent with the biochemical
findings, obvious hepatocyte apoptosis was observed by TUNEL staining in marginal copper deficient rats fed with fructose, as seen in representative photomicrographs of TUNEL staining (Figure 3b). In addition, fructose feeding plus marginal copper deficiency increased plasma MCP-1 level compared to marginal copper deficient rats and adequate copper rats fed with fructose (Figure 3c).

Dietary marginal copper deficiency and low fructose ingestion synergistically accelerate hepatic fat accumulation
Neither adequate copper with fructose in the drinking water nor marginal copper deficiency alone significantly increased hepatic...

![Graphs showing liver iron and plasma ferritin levels](image)

**FIGURE 2** Effect of low fructose feeding on iron status. (a) Liver iron. (b) Plasma ferritin. Data represent means ± SD (n = 5-10). *P < 0.05. A, adequate copper diet; M, marginal copper deficient diet; AF, adequate copper diet + 3% fructose drinking; MF, marginal copper deficient diet + 3% fructose drinking.

![Graphs showing plasma ALT and AST levels](image)

**FIGURE 3** Effect of marginal copper deficiency and low fructose feeding on liver injury. (a) Plasma ALT and AST levels. (b) Representative photomicrographs of TUNEL staining of liver section (400×). (c) Plasma MCP-1. Data represent means ± SD (n = 5-10). *P < 0.05; #, interaction between copper and fructose is significant (P < 0.05). A, adequate copper diet; M, marginal copper deficient diet; AF, adequate copper diet + 3% fructose drinking; MF, marginal copper deficient diet + 3% fructose drinking. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; MCP-1, monocyte chemoattractant protein-1.
triglyceride content compared to adequate copper rats after 4 weeks. However, fructose drinking combined with marginal copper deficiency significantly and synergistically induced hepatic triglyceride accumulation (Figure 4b). Those changes were also consistent with histology as shown by H&E staining (Figure 4a).

**Decreased antioxidant defense system and increased oxidative stress by dietary marginal copper deficiency and low fructose ingestion**

We next examined the effects of marginal copper deficiency and fructose ingestion on oxidative stress, including GSH/GSSG and 4-HNE. Hepatic GSH and GSSG levels were significantly decreased by fructose drinking in either marginal copper deficiency or adequate copper rats. The GSH/GSSG ratio, which is an indicator of oxidative stress, was significantly increased in both adequate and marginal copper deficient rats when fed with fructose compared to controls. Fructose feeding led to a further increased GSH/GSSG ratio in marginal copper deficient rats compared to adequate copper rats (Figure 5a). As shown in Figure 5b, the representative photomicrographs of liver 4-HNE immunohistochemistry staining, immunoreactivity of 4-HNE was slightly increased in the livers of adequate copper rats fed with fructose, and it was significantly enhanced in marginal copper deficient rats fed with fructose compared with controls, suggesting increased lipid peroxidation and oxidative stress in marginal copper deficient rats fed with fructose.

**Discussion**

In this animal study, we used 3% fructose (w/v) as the modest fructose feeding, which is much lower than the 30% fructose (w/v) we used previously (21). Actually, 3% fructose (w/v) in the drinking water is very close to the typical HFCS-55 consumption as sweetened beverages in the current American diet (29,30).

It is well known that the etiology of NAFLD is complex, and multiple factors are likely involved, including nutritional and genetic factors. Recently, decreased copper availability was shown in NAFLD patients (15,16). Interestingly, decreased copper availability may not only be because of inadequate intake of food, but may also be because of dietary high fructose, which might impair copper absorption from the intestine (17-21). In this study, we report for the first that modest fructose intake and marginal copper deficiency together induced liver injury and fat accumulation in rats. The fructose concentration in this study is only about 10% of that in the high fructose drinking, as was used in our previous study. While there is a ten-fold difference in the amount of fructose in the drinking water, the estimated fructose absorption is already saturated even at 3% fructose (23,24). Therefore, it is plausible that 3% and 30% fructose (w/v) interfere with copper absorption and impair copper status to a similar level, suggesting that marginal copper deficiency may play an important role in the pathogenesis of NAFLD. Further, decreased copper availability might be a critical factor underlying fructose-induced NAFLD.

Modest fructose consumption did not significantly increase hepatic triglyceride content in adequate copper rats in 4 weeks. However, it significantly increased hepatic triglyceride content in marginal copper deficient rats by approximately three folds during that same time. In our previous study, high fructose feeding (30% fructose, w/v) increased hepatic triglyceride content by four folds in marginal copper deficient rats (21). Plasma AST was increased by five folds in marginal copper deficient rats when feeding 30% fructose (21), and it increased by two folds when animals were fed with 3% fructose. Compared to our previous data from 30% fructose drinking, it appears that increased fat accumulation and liver injury do not parallel the
amount of fructose consumption, but seems more likely to relate to the copper status. In fact, previous studies have reported that dietary fructose impaired copper absorption from the intestine (31,32), potentially by blocking copper transport through copper transporter-1 (21).

Hepatic iron overload is a common feature associated with copper deficiency (33,34). Our data clearly showed increased liver iron content in marginal copper deficient rats even with modest fructose consumption (Figure 2a). Basically, excess iron was diffusely distributed throughout hepatocytes and a large portion within Kupffer cells (resident macrophages in liver) (35). Macrophages play a crucial role in iron recycling by phagocytosing senescent erythrocytes and releasing accumulated iron back into the circulation in a regulated manner, thus enabling iron recycling. One important mechanism underlying copper deficiency associated hepatic iron overload is decreased plasma ceruloplasmin, which regulates iron efflux from reticuloendothelial cells, including liver Kupffer cells via ferroxidase (33). Down-regulation of the iron exporter, ferroportin-1 (Fp-1) (36), or some other unknown mechanism may also contribute to the iron accumulation.

One mechanism underlying fructose-induced liver injury in marginal copper deficient rats is decreased antioxidant defenses caused by copper deficiency and increased oxidative stress. In this study, decreased antioxidant defense is characterized by decreased GSSG in animals fed with fructose (which has already been observed previously by our group), possibly because of the down-regulated glutathione peroxidase (GPx) (21). Therefore, GSH cannot be fully oxidized to remove hydrogen peroxide and cannot perform its antioxidant function. However, fructose feeding did not lead to liver injury in adequate copper rats, while it did in the marginal copper deficient animals, suggesting other factors associated with copper deficiency might play a role. In fact, decreased copper, zinc-superoxide dismutase (SOD1) as a result copper deficiency is well documented in previous studies, including from our group, and may be another important factor contributing to decreased antioxidant defense (21,37). Thus, it is likely that decreased antioxidant defense as a result of dysregulation of the GSH/GSSG system and suppressed SOD1 because of copper deficiency may led to increased accumulation of superoxide and hydrogen peroxide. On the other hand, hepatic iron overload is a common feature associated with copper deficiency (33,34), and hepatocytes are important cells for iron accumulation (35). Iron is a catalyst to convert hydrogen peroxide to hydroxyl radicals through the Haber-Weiss and Fenton reactions. Therefore, ROS generated from the mitochondrial respiratory chain may be further increased by iron overload, subsequently leading to hepatocyte cell death/liver injury.

In conclusion, our data clearly showed that 3% fructose as the added sugar beverage further impaired copper status and led to significant hepatic fat accumulation and liver injury in marginal copper deficient rats, suggesting that dietary fructose and copper interaction may play an important role in the pathogenesis of fructose-induced NAFLD, and marginal copper deficiency might be an important priming factor in the “two hit” model of NASH. One potential mechanism underlying copper deficiency-induced fatty liver is likely because of hepatic iron overload. The pathogenesis of NAFLD is a complicated process and likely involves multiple factors. Our data provide strong evidence in understanding the pathogenesis of NAFLD and potential new therapeutic targets for the future.

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