Research Paper

An iron-deficient diet prevents alcohol- or diethylnitrosamine-induced acute hepatotoxicity in mice by inhibiting ferroptosis

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1. Introduction

Exogenous chemicals, such as pharmaceuticals, herbas, and environmental pollutants, can trigger acute liver injuries, in the worst-case acute liver failure, and may develop into hepatic steatosis, cholestasis, necrosis, and cirrhosis (Stravitz and Lee, 2019; SK et al., 2019; RJ et al., 2019; VictorNavarro et al., 2017). Furthermore, these liver-damaging chemicals could exacerbate liver injuries induced by other pathogenic factors, e.g. viral hepatitis (Vandenbulcke et al., 2016). Exogenous chemicals-induced liver injuries have been regarded as a serious health problem, hence its prevention and management raise worldwide interest (Meng et al., 2018).

Reactive oxygen species (ROS) are frequently involved in liver injuries triggered by exogenous chemicals, which react with and disable biomacromolecules such as lipids, proteins, or DNA. Ferroptosis is a recently defined, iron-dependent cell death mechanism characterized by iron accumulation and lipid peroxide accumulation (Dixon et al., 2012; Tang et al., 2020a). Many findings indicate that ferroptosis participates in various liver diseases including hepatitis, fatty liver, hepatocellular carcinoma, as well as liver injuries triggered by exogenous chemicals (Capelletti et al., 2020; Jiang et al., 2021; Stockwell et al., 2017a). Hepatocytes undergoing ferroptosis exhibit an increase in malondialdehyde (MDA) content, an end-product of lipid peroxidation, depletion of glutathione (GSH), exhaustion of the antioxidative system, and changes in expressions of glutathione peroxidase 4 (GPX4), which eliminated malondialdehyde (MDA) by utilizing glutathione (GSH). In summary, alcohol- or DEN-induced liver injuries were mitigated by the iron-deficient diet by inhibiting ferroptosis, which might be a promising measure for preventing liver injuries induced by alcohol, DEN, or other exogenous chemicals.

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lipids nonenzymatic peroxidation and ferroptosis (Jiang et al., 2021). Besides, iron is the catalytic factor for many oxidoreductases involved in polyunsaturated fatty acids peroxidation, such as ALOXs and NOXs (Stockwell et al., 2017a). A lot of studies have revealed that iron chelators inhibit ferroptosis to exert therapeutic or preventive effects in neurodegeneration (Masaldan et al., 2019), hemochromatosis (Wang et al., 2017), nonalcoholic steatohepatitis (Tsurusaki et al., 2019), atherosclerosis (Bai et al., 2020), and so on. Inducing ferroptosis in malignant cells is also a hot topic in cancer therapy (Hassannia et al., 2019). However, it is currently unknown whether iron restriction could prevent exogenous chemicals-induced liver injuries by inhibiting ferroptosis.

Here, we used an iron-deficient diet to induce iron deficiency in mice (Li et al., 2022). Then, alcohol and DEN were used to mimic mild liver injury and severe liver failure respectively. The effects of the iron-deficient diet on protecting the liver from alcohol- or DEN-induced injuries were evaluated. Underlying mechanisms including drug metabolism and ferroptosis were also determined. These results would provide experimental evidence for preventing liver injuries from exogenous chemicals through an iron-deficient diet.

2. Materials and methods

2.1. Animal studies

Animal studies were performed under the approval of the Institutional Animal Care and Use Committee of Second Military Medical University, following the guidance of “Guide for the Care and Use of Laboratory Animals”. Male C57BL/6 mice (8 weeks of age) were purchased from the SLAC Laboratory Animal, Inc., Shanghai, China, and reared in SPF grade environment in the Animal Center of Second Military Medical University, with normal circadian rhythm and food or water ad libitum. Production of the iron control diet and iron-deficient diet was commissioned to Trophic Animal Feed High-Tech Co., Ltd (Nantong, China), according to the AIN-93 formula. Iron content is about 45 mg/kg in the iron control diet, and <6 mg/kg in the iron-deficient diet, as confirmed by flame atomic absorption detection. After four weeks of feeding, mice were treated with three times alcohol (5 g/kg, 24 h interval, gavage) to mimic mild liver damage (Ambade et al., 2014) (n = 6 in each group), and five times DEN (50 mg/kg, 24 h interval, i. p. injection) to mimic acute liver failure (Fuentes-Hernandez et al., 2019) (n = 10 in each group). Mice in the control group were treated with the same dose of PBS. The experimental streamline could be seen in Fig. 1.

2.2. Determination of serum iron content and ALT, AST

The serum iron concentration and serum contents of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by Hitachi 7600 Automatic Biochemical Analyzer (Hitachi, Tokyo, Japan) as before (Wang et al., 2022).

2.3. Determination of liver AHD and ALDH activities and GSH and MDA contents

Liver tissues were homogenized and centrifuged to obtain the supernatant. The ADH activities were determined by a Micro Alcohol Dehydrogenase (ADH) Assay Kit (Cat. BC0185, Solarbio Life Science, Beijing, China). The ALDH activities were determined by a Micro Acetaldehyde Dehydrogenase (ALDH) Assay Kit (Cat. BC0755, Solarbio Life Science, Beijing, China). The GSH content was determined by a Micro Reduced Glutathione (GSH) Assay Kit (Cat. BC175, Solarbio Life Science, Beijing, China). The MDA content was determined by a Micro Malondialdehyde (MDA) Assay Kit (Cat. BC0025, Solarbio Life Science, Beijing, China).

2.4. Western Blot analysis

Western Blot assays were performed and analyzed as before (Tang et al., 2020b). Briefly, a whole extract was prepared by a Total Protein Extraction Kit (Keygene Biotech, Nanjing, China). Following normalization of protein concentration by using BCA kits (Thermo Fischer, US), the samples were separated in 10% SDS-PAGE electrophoresis and electrotransferred to a nitrocellulose membrane. Validated antibodies used in this study were: CYP2E1 (1:500 Cat. D122177, Sangon Biotech, China), GPX4 (1:1000, Cat. MAB5457, Bio-techne, USA), β-actin (1:2000, Cat. D110001, Sangon Biotech). The IRDye® secondary antibody (1:10,000, LI-COR, USA) was used and immunoblots were scanned by the Odyssey® dual-color infrared fluorescence imaging system. The grayscale of each band was obtained from Odyssey® software.

2.5. Statistics

Data were presented as Mean ± SD. One-way ANOVA was used for

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**Fig. 1. Experimental design.** (A) Model of alcohol-induced liver damages: 8 weeks old male mice were fed an iron-deficient diet for four weeks. Weights and food consumption were recorded twice a week. Then mice were intragastrically administered with 5 g/kg alcohol for 3 times, 24 h interval. Weights and food consumption were recorded every time. 24 h after the last gavage, mice were euthanized for collecting serum and liver tissues. (B) Model of DEN-induced liver damages: 8 weeks old male mice were fed an iron-deficient diet for four weeks. Weights and food consumption were recorded twice a week. Then mice were i. p. injected with 50 mg/kg DEN for 5 times, 24 h interval. Weights and food consumption were recorded every time. 24 h after the last injection, mice were euthanized for collecting serum and liver tissues.
multigroup comparison. Tukey’s multiple comparisons test was used for post hoc tests. The difference was significant when p < 0.05.

3. Results

3.1. Overall status and iron contents in mice

As exhibited in Fig. 1, the weights of mice and food consumption were recorded during the experimental period. Mice that received four weeks of iron-deficient diet (ID) did not show differences in weights compared to mice that received the control diet (Con) (Fig. 2A). However, the gavage of alcohol or PBS decreased the weights of mice (Fig. 2A), probably due to the gavage decreasing the food intake of mice (Fig. 2B). At the end of the experiment, serum and liver tissues were collected for iron content determination. The results showed that the serum iron content (Fig. 2C) and liver iron content (Fig. 2D) were significantly decreased by the iron-deficient diet. The acute treatment of alcohol did not affect serum and liver iron contents. Likewise, the weights of mice in the DEN experiment were not changed by the iron-deficient diet, but the i. p. Injection of DEN decreased the iron contents (Fig. 2E). The food intake also decreased significantly by DEN injection (Fig. 2F). The serum iron content (Fig. 2G) and liver iron content (Fig. 2H) were decreased by the iron-deficient diet, while the DEN treatment also decreased the liver iron content of mice receiving the control diet (Fig. 2H).

3.2. Iron deficiency inhibited hepatotoxicity of alcohol or DEN

After validation of iron deficiency in mice receiving the iron-deficient diet, the effects of iron deficiency on alcohol- or DEN-induced liver injuries were evaluated. The hepatocytes morphology was characterized by H&E staining (Fig. 3A & E). The acute alcohol treatment led to significant vacuolation in hepatocytes with shrinking liver parenchyma (Fig. 3A), which were alleviated by the iron-deficient diet. The liver/body weight ratio (Fig. 3B), serum ALT (Fig. 3C), and serum AST (Fig. 3D) that were increased by alcohol treatment were also prevented by the iron-deficient diet. In DEN-treated mice, the hepatocytes showed more severe morphological changes including cell swelling, hyaline degeneration, binucleate cells, and lysis cells (Fig. 3E). The iron-deficient diet also alleviated these changes in hepatocytes, although the effects were not much as in alcohol-treated mice. The liver/body weight ratio (Fig. 3F), serum ALT (Fig. 3G), and serum AST (Fig. 3H) were also decreased by the iron-deficient diet in mice treated with DEN. These results suggested that the acute hepatotoxicity of alcohol or DEN could be prevented by an iron-deficient diet.

3.3. Iron deficiency increased the activities of metabolic enzymes of alcohol and DEN

The activities of alcohol dehydrogenase (ADH), the main metabolic enzyme of alcohol, were decreased by alcohol treatment (Fig. 4A). Interestingly, this decrease was prevented by iron deficiency (Fig. 4A). The activities of acetaldehyde dehydrogenase (ALDH) were not affected by alcohol treatment but increased by iron deficiency (Fig. 4B). The expression of CYP2E1, the main metabolic enzyme of DEN, was dramatically decreased by DEN treatment and increased by iron deficiency in the liver (Fig. 4C). These results suggested that the metabolism of alcohol and DEN was enhanced by iron deficiency.

3.4. Iron deficiency mitigated alcohol or DEN-induced ferroptosis by preventing GPX4 decrease

The hepatic MDA content was increased by the acute administration of alcohol (Fig. 5A) and the increase was stronger in the severe liver failure induced by DEN (Fig. 5D), implying that ferroptosis was involved in alcohol or DEN-induced liver injuries. In the mice of acute alcohol treatment, expression of GPX4 was decreased in the liver, accompanied by the elevation of hepatic GSH content (Fig. 5B & C). In contrast, iron deficiency could effectively prevent the decrease of GPX4, which utilized GSH to reduce peroxidative lipids (Fig. 5B & C). In mice treated with DEN, the hepatic MDA content rose dramatically and the expression of GPX4 sharply decreased (Fig. 5E). Iron deficiency also prevented the decrease of GPX4 expression and increased the GSH utilization in the liver of DEN-treated mice (Fig. 5E & F), suggesting that iron deficiency could alleviate lipid peroxidation by protecting the GPX4-GSH antioxidative system.

Fig. 2. Overall status and iron contents in mice treated with alcohol or DEN after feeding an iron-deficient diet for four weeks. (A) Weights, (B) Daily food intake, (C) Serum iron concentration, and (D) Liver iron contents of mice received control diet (Con) or iron-deficient diet (ID) and treated with three times of alcohol (5 g/kg, 24 h interval, gavage, n = 6). (E) Weights, (F) Daily food intake, (G) Serum iron concentration, and (H) Liver iron contents of mice received control diet (Con) or iron-deficient diet (ID) and treated with five times DEN (50 mg/kg, 24 h interval, i. p. Injection, n = 10). *: p < 0.05, **: p < 0.01, ***: p < 0.001, one-way ANOVA with Tukey’s multiple comparisons tests.
4. Discussion

In the present study, we focused on the role of iron deficiency in preventing acute liver injuries induced by two hepatotoxic chemicals, alcohol and DEN. The results indicated that iron deficiency effectively inhibited liver injuries by restoring GPX4 expression and mitigating ferroptosis (Fig. 6).

Alcohol and its primary metabolic product acetaldehyde generate ROS in cells, leading to peroxidation of lipids and adducts of protein and DNA (HK et al., 2018). The alcohol-induced hepatotoxicity is amplified by exogenous iron (Do et al., 2013). Even under low alcohol intake, a certain amount of iron overload can cause significant liver oxidative damage (Gao et al., 2017), introducing a synergistic interaction of alcohol and iron. On the contrary, maintaining intracellular liable iron pool protects hepatocytes from oxidative damage induced by alcohol (Li et al., 2014). We found that the iron-deficient diet could prevent liver injuries induced by alcohol. The lipid peroxidation and ferroptosis induced by alcohol were alleviated by iron deficiency. ADH oxidizes alcohol to acetaldehyde, which is further oxidized by ALDH to acetic acid (Louvet and Mathurin, 2015). The activities of ADH and ALDH were increased by iron deficiency, meaning that alcohol metabolism in the liver was accelerated. Likewise, the activities of CYP2E1, the enzyme that mediates the production of ROS in DEN metabolism (Gao et al., 2018), were increased by iron deficiency. Higher CYP2E1 activity correlates with oxidative damages (Martinez-Gil et al., 2020), inflammation, hepatofibrosis (Guo et al., 2019), and hepatocarcinogenesis induced by DEN (Gao et al., 2018). Although the increased activities of CYP2E1 enhanced the transformation of DEN to phase II reaction, the simultaneous production of ROS led to oxidative damage. In fact, it was proved that cytochrome P450 oxidoreductase contributes to phospholipid peroxidation in ferroptosis (Zou et al., 2020). While the metabolism of alcohol or DEN was accelerated by iron deficiency, with more production of ROS, the liver injuries and lipid peroxidation induced by alcohol or DEN were prevented by iron deficiency, suggesting that iron deficiency protected the liver by inhibiting ferroptosis, rather than accelerating the metabolism of alcohol or DEN.
A recent study indicated that ferroptosis was involved in alcohol-induced cell death in vivo and in vitro (Liu et al., 2020b). Likewise, DEN could induce early oxidative stress and ferroptosis in the rat liver (Mansour et al., 2019). In line with these, our results also showed that alcohol or DEN treatment induced ferroptosis in the liver of mice. Ferroptosis requires the participation of iron and the inactivation of GPX4, which reduces phospholipid hydroperoxides and prevents lipid peroxidation (Stockwell et al., 2017b; Seibt et al., 2019). Iron deficiency may inhibit ferroptosis through: (1) limiting available ferrous iron that catalyzes the production of hydroxyl radical; (2) reducing the activities of oxidases for poly-unsaturated fatty acids, like ALOXs and NOXs, which need iron as a co-factor. In the present study, we found that iron deficiency increased GPX4 expression in the liver of alcohol or DEN-treated mice to reduce lipid peroxidation by using GSH. Loss of GPX4 expression is widely observed in ferroptosis (Maiorino et al., 2018). GPX4 inhibitors, such as ML-210 and RSL3, could directly induce ferroptosis in various cell types (Soula et al., 2020; Jia et al., 2020). So far, there has been little evidence hinting that iron chelators may increase GPX4 activities and expression (Ma et al., 2020; Zhang et al., 2021; Meng et al., 2019). Our present study pointed out that an iron-deficient diet prevented the decreased GPX4 expression by alcohol or DEN. However, the iron deficiency itself did not affect the expression of GPX4, the underlying mechanisms need further exploration and elucidation.

Iron chelators have been used as a therapeutic measure in iron overload diseases, especially in patients with hemochromatosis or frequent blood transfusions (Taccone-Gallucci et al., 2011). The ability to inhibit ferroptosis gives iron chelators more applicable fields, including cancers, cardiovascular and cerebrovascular diseases, neurodegeneration diseases, and exogenous chemicals-induced cell injuries (Zhou et al., 2020; Bai et al., 2020; Su et al., 2020; Lorincz et al., 2015; JM and M, 2014). Unfortunately, iron chelators may bring untoward effects that limit their applications, such as anemia, urticaria, hypotension, nephrotoxicity, olfactory dysfunction, gastrointestinal dysfunction, neutropenia, and agranulocytois (Derin et al., 2019; Mody et al., 2019). Thus the seeking of low-toxic iron chelators or alternative measures, such as exogenous transferrin, exogenous hepcidin, hepcidin analogues, and hepcidin signaling agonists, continues (Fleming and Ponka, 2012). Recent studies reported that novel iron chelators like CN128 (Sun et al., 2020; Chen et al., 2020), Quillamine HQ1-44 (Renaud et al., 2015), 1-(N-acetyl-l-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (Pangit et al., 2015), and some phytochemicals like curcuminoids (Jiao et al., 2009) and quercetin (Horniblow et al., 2017), showed protective functions in many diseases by chelating iron, with no or little side effects. Our study highlighted the use of an iron-deficient diet to induce iron deficiency in restricting ferroptosis and preventing liver injuries, which was harmless than iron chelators because the non-existed side effects and the degree of

Fig. 5. Iron deficiency prevented the decrease of GPX4 to alleviate ferroptosis. (A) Liver MDA content, (B) GPX4 expression, and (C) Liver GSH content in mice received the iron-deficient diet (ID) and treated with three times alcohol (5 g/kg, 24 h interval, gavage, n = 6). (D) Liver MDA content, (E) GPX4 expression, and (F) Liver GSH content in mice received the iron-deficient diet (ID) and treated with five times DEN (50 mg/kg, 24 h interval, i. p. Injection, n = 10). *: p < 0.05, **: p < 0.01, ***: p < 0.001, one-way ANOVA with Tukey’s multiple comparisons tests.

Fig. 6. Exogenous chemicals like alcohol and diethylnitrosamine (DEN) induce acute liver injuries through lipid peroxidation and ferroptosis of hepatocytes. An iron-deficient diet prevents alcohol- or DEN-induced ferroptosis by upregulating the expression of GPX4 to utilize GSH, thus eliminating lipid peroxidation, although it also accelerates the metabolism of alcohol and DEN. ADH: alcohol dehydrogenase; ALDH: acetaldehyde dehydrogenase; CYP2E1: Cytochrome P450 Family 2 Subfamily E Member 1.
iron deficiency would not be overwhelmed, for the reason that the iron deficiency induced by iron-deficient diet will increase the iron absorption and the majority of iron source is from the recycle use of iron in aged erythrocytes. Therefore, the iron-deficient diet is more suitable in male adults, with the higher prevalence of liver diseases than women (Asrani et al., 2019), rather than children or pregnant women who are vulnerable to iron-deficient anemia.

In conclusion, our results introduced the protective effects of an iron-deficient diet on liver injuries induced by alcohol or DEN, by upregulating GPX4 expression to inhibit ferroptosis. The convenience, inexpensiveness, and low toxicity enable the iron-deficient diet to be a promising preventive measure for exogenous chemicals-induced liver injuries. Due to the limitations of the present study that focused on acute liver injuries, the protective effects of iron-deficient diet on chronic liver injuries including alcoholic fatty liver diseases and non-alcoholic fatty liver diseases need to be further explored.

CRediT authorship contribution statement

Zelong Gao: Investigation, Visualization, Writing – original draft.
Dongyao Wang: Investigation, Validation, Writing – original draft.
Hongwei Zhang: Investigation, Validation. Jianxin Yang: Methodology, Resources. Min Li: Supervision, Writing – review & editing. Hongtao Lu: Methodology, Resources. Hui Shen: Supervision, Writing – review & editing. Yuxiao Tang: Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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