Preventive effects of lactoferrin on acute alcohol-induced liver injury via iron chelation and regulation of iron metabolism

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ABSTRACT

Lactoferrin is widely found in milk and has the ability to bind iron. Previous studies have reported that lactoferrin was effective in the prevention and treatment of acute alcohol-induced liver injury (AALI). Ferroptosis is a recently discovered cell death and is involved in the development of AALI. However, the potential role of lactoferrin in acute alcohol-induced ferroptosis is still unclear. In this study, we observed that lactoferrin (10, 20 and 40 μg/mL) significantly mitigated alcohol (300 mM)-induced injury in vitro. Additionally, lactoferrin (100 and 200 mg/kg bw) significantly alleviated alcohol (4.8 g/kg bw)-induced injury in vivo. Our results showed that lactoferrin inhibited alcohol-induced upregulation of the ferroptosis marker protein ACSL4 and downregulation of GPX4. Meanwhile, lactoferrin treatment successfully reversed the elevated Malondialdehyde (MDA) levels and the reduced Glutathione (GSH) levels caused by alcohol treatment. These results can indicate that lactoferrin significantly decreased ferroptosis in vivo and in vitro. Lactoferrin has the potential to chelate iron, and our results showed that lactoferrin (20 μg/mL) significantly reduced iron ions and the expression of Ferritin Heavy Chain (FTH) under FeCl3 (100 μM) treatment. It was demonstrated that lactoferrin had a significant iron-chelating effect and reduced iron overload caused by FeCl3 in AML12 cells. Next, we examined iron content and the expression of iron metabolism marker proteins Transferrin Receptor (TFR), Divalent metal transporter 1 (DMT1), FTH, and Ferroportin (FPN). Our results showed that lactoferrin alleviated iron overload induced by acute alcohol. The expression of TFR and DMT1 was downregulated and FPN and FTH were upregulated after lactoferrin treatment in vivo and in vitro. Above all, the study suggested that lactoferrin can alleviate AALI by mitigating acute alcohol-induced ferroptosis. Lactoferrin may offer new strategies for the prevention or treatment of AALI.

Key words: lactoferrin, alcohol, ferroptosis, iron metabolism

INTRODUCTION

Lactoferrin is a multifunctional protein with multiple biological activities and applications (González-Chávez et al., 2009). Lactoferrin belongs to the transferrin family and is classified as an iron-binding protein by chelating free Fe3+ and Fe2+ ions (Superti, 2020). Therefore, lactoferrin has a high affinity for iron ions and regulates free iron levels (Baker and Baker, 2004). Several studies confirm that lactoferrin can protect the liver injury caused by acetaminophen, concanavalin A and carbon tetrachloride (Yin et al., 2010b; a; Farid et al., 2021). Meanwhile, lactoferrin positively regulates lipid metabolism and ameliorates hepatic steatosis in obese mice (Guo et al., 2020). Notably, previous studies have demonstrated the preventive effect of lactoferrin on acute alcohol-induced liver injury in mice (Li et al., 2021, 2022a). Therefore, lactoferrin has great potential as a natural dietary ingredient in the treatment of AALI. Alcohol is one of the extensively consumed drinks globally, still poses a major threat to public health and contributes significantly to the occurrence of illness and death worldwide (Luo et al., 2023). AALI is one of the typical alcohol liver diseases, caused by excessive drinking over a short period. AALI shares a complex pathogenesis and the exact mechanism is not completely clear (Sugimoto and Takei, 2017). It is noteworthy that iron overload and oxidative stress in the liver are critical features of alcoholic liver injury (Li et al., 2022b). Our previous study showed that ferroptosis was involved in alcohol-induced liver injury (Zhao et al., 2021). Mean-
while, iron overload has been found to worsen alcoholic liver injury through 2 mechanisms: increasing alcohol-induced oxidative stress and inducing ferroptosis in the liver (Harrison-Findik et al., 2006; Ali et al., 2023). Therefore, targeting the process of ferroptosis may be the potential to be a valuable approach in preventing or mitigating alcohol-induced liver injury.

Ferroptosis is a recently discovered iron-dependent regulation of cell death (Tang and Kroemer, 2020). Due to the involvement of the ferroptosis response in multiple pathophysiological processes, it has received widespread attention. Lipid peroxidation due to excess iron is a direct stimulus for ferroptosis (Yu et al., 2020; Chen et al., 2022). Therefore, it is essential to focus on maintaining intracellular iron homeostasis to alleviate ferroptosis and AALI. The most frequent methods to target iron homeostasis include iron chelation and iron metabolism. Research has suggested that the ability of lactoferrin to chelate iron was critical to its many biological roles and the ability to scavenge iron helped reduce oxidative stress and inflammatory responses (Legrand, 2016). Notably, it has been demonstrated that lactoferrin had a preventive effect on alcohol-induced liver injury, with potential mechanisms related to elevated redox-stress response capacity (RRC), regulation of hepatic alcohol metabolism, redox homeostasis, and lipid metabolism (Li et al., 2021, 2022a). However, there are fewer studies on lactoferrin in ferroptosis, and the mechanism of lactoferrin in ferroptosis is not completely clear.

Dairy products, being an integral part of the human diet, generate numerous by-products throughout their production and processing. These by-products encompass a diverse range of ingredients, including whey protein, casein, lactoferrin, and others, which possess significant biological properties (Svanborg et al., 2015). Evaluating and effectively utilizing these active ingredients can enhance the value of the dairy industry. Lactoferrin has garnered significant attention and research focus owing to its functional activity and safety. This study aimed to explore the protective effects and mechanisms of lactoferrin against acute alcohol-induced ferroptosis in vivo and in vitro. We suggest that lactoferrin alleviates ferroptosis by iron chelation and regulating cellular iron metabolism, thereby alleviating liver injury. In conclusion, our study contributes to a better understanding of the specific role and mechanism of lactoferrin in AALI.

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Animal Experiments

Male C57BL/6J mice were obtained from Liaoning Changsheng Biotechnology (Shenyang, China). The ages of the mice ranged from 6 to 8 weeks, and their weight varied between 18 to 22 g. To ensure consistent conditions, the mice were housed in a controlled SPF chamber, wherein the temperature was maintained at 23 ± 2°C, and the relative humidity was kept between 50 and 60%. The housing followed a 12:12-h light-dark cycle during the entire duration of the study. All animal procedures and protocols in this study were approved by the Animal Experimentation Committee of Jilin University (approval number: SY202306027). Randomly dividing the mice into 4 groups of 6 mice each, the study incorporated the control group, model group, low-dose lactoferrin treatment group, and high-dose lactoferrin treatment group. To determine the dosage of lactoferrin, we took into consideration the recommended daily intake as well as previous research on the protective effects of lactoferrin on the liver. The doses we selected for this study were 100 mg/kg bw and 200 mg/kg bw, which are lower than those used in previous studies but still have significant effects (Li and Hsieh, 2014; Aoyama et al., 2022; Hu et al., 2023). Therefore, the lactoferrin treatment group received the appropriate quantity of lactoferrin by gavage, while the control and model groups received the same amount of saline by gavage every day for 4 weeks (Tung et al., 2014; Li et al., 2021; Zhang et al., 2024). After 4 weeks of feeding, AALI was induced by intragastric administration of alcohol (4.8 g/kg bw) every 12 h for 3 continuous sessions and anesthesia with mild isoflurane (Li et al., 2021). Once the animals were sacrificed, blood samples were obtained and allowed to coagulate at ambient temperature for a duration of 30 min. Subsequently, the blood specimens underwent analysis to ascertain the quantities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) following centrifugation at a speed of 3500 rpm for 10 min at a
temperature of 4°C. A segment of the liver tissue was retained in a solution consisting of 10% formalin to facilitate hematoxylin-eosin (H&E) staining, whereas the remaining portion was promptly frozen to facilitate subsequent analysis (Zhao et al., 2022).

**Cell Culture**

Cell culture was performed as previously described (Liu et al., 2023; Meng et al., 2023). Briefly, Shanghai Institute of Biochemistry and Cell Biology (SIBCB, Shanghai, China) provided AML-12 cells. We used Dulbecco’s modified Eagle medium/F-12 (DMEM/F-12) from Gibco (11330500) to culture AML-12 cells. This medium is commonly used for the growth and maintenance of these cells. DMEM/F-12 lacks proteins, lipids, or growth factors and therefore requires the addition of 10% fetal bovine serum (Clark Bioscience, FB15015), 1% ITS liquid medium supplement (Sigma, I3146), 1% 100 μg/mL penicillin and streptomycin (HyClone, SV30010) and 40 ng/mL dexamethasone under culture conditions of 37°C, 5% CO2 when used in cell culture (Meng et al., 2023). AML12 cells were used at the confluence of 80% before contact treatment in the experiments. Based on the results of the cell viability assay and previous studies, we determined the treatment doses of lactoferrin and deferoxamine (Ma et al., 2020; Liu et al., 2023). The AML12 cells were addressed with lactoferrin (10, 20, or 40 μg/mL) and deferoxamine (DFO, 10μM) for 2 h and followed with a treatment of alcohol (300 mM) for another 22 h or FeCl3 (100 μM) for another 3 h, and the cells were collected for the analysis of proteins.

**Cell Viability Assay**

AML12 cells were grown in 96-well plates for a duration of 24 h with a cell density of 1 × 10^4 cells per well (Zhao et al., 2021). Following this, different concentrations of LF (0, 20, 40, 60, 80, and 100 μg/mL) and alcohol (300 mM) were administered to the cells either individually or as a mixture for an additional 24 h. Next, the plate was subjected to a 4-h incubation period with MTT at a concentration of 0.5 mg/mL. Subsequently, the resulting formazan crystals were dissolved in a 150 μL solution of DMSO. Finally, the optical density (OD) at a wavelength of 570 nm was measured using a microplate reader (Bio Tek).

**Detection of ROS and Lipid ROS**

The pretreatment of AML-12 cells was carried out after their inoculation into 24-well plates. Following that, the cells were subjected to staining using BODIPY™ 581/591 C11 (Thermo Fisher, D3861). The incubation process was performed in darkness at a temperature of 37°C for a duration of 30 min. To determine intracellular levels of reactive oxygen species (ROS), a fluorescent ROS assay kit (Beyotime, S0033S) was employed. The cells were rinsed 3 times with 1 × PBS and then visualized with an FV31S-SW fluorescence microscope (Olympus, Tokyo, Japan) and AE31 fluorescence microscope (Olympus, Tokyo, Japan).

**Contents of AST, ALT, Iron, Glutathione (GSH), and Malondialdehyde (MDA)**

The assay kits provided by Nanjing Jiancheng Bio Co., Ltd. (A003–4–1, A005–1–2) were used to determine the levels of MDA and GSH in mice liver and AML12 cells. The assay kits provided by Nanjing Jiancheng Bio Co., Ltd. (C010–2–1, C009–2–1) were used to determine the levels of AST and ALT in the serum of mice. The assay kits provided by APPLYGEN (E1042–100) were used to determine the levels of iron in mice liver and AML12 cells.

**Western Blot Analysis**

The expression levels of relevant proteins under different treatments can be detected using Western blotting. The cells were gathered and subjected to lysis using a cell lysis solution (Beyotime, P0013), followed by a 30-min ice bath while being mixed. After that, we quantified the protein samples extracted from AML12 cells using a BCA assay kit (Beyotine, P0009) to ensure that the total protein concentration was the same for each set of protein samples. Proteins were separated using a 6–12% SDS-PAGE technique. Then, semi-dry blotting was employed to transfer these proteins onto polyvinylidene difluoride (PVDF) membranes. To avoid non-specific binding, the PVDF membranes underwent a 2-h incubation at room temperature with 5% bovine albumin, while gently shaking. Subsequently, the primary antibody was incubated with the membrane overnight at 4°C, followed by 3 rinses using TBST. Then, the membrane was incubated with the secondary antibody at room temperature for 2 h, while being gently shaken. The visualization of bands was performed using an electrochemiluminescence system (Azure C300, USA). Protein expression levels were quantified using the ImageJ software.

**Prussian Blue Staining**

A density of 3–4 × 10^5 cells was used to inoculate 6-well plates. The cells underwent pretreatment before further procedures. Following the treatment, a thorough
rinsing with PBS was performed, followed by fixation with a solution of 4% paraformaldehyde for 30 min. Subsequently, the cells were stained with Prussian blue (APPLYGEN, Beijing, China, B1113) for a duration of 30 min. Once stained, a washing step with PBS was carried out, followed by staining with nuclear solid red to identify nuclear structures. Samples were observed by microscope AE31 (Olympus, Tokyo, Japan).

**FerroOrange Staining**

AML-12 cells were treated for 30 min at 37°C using FerroOrange (MKBio, Shanghai, China, MX4559). After the cells were washed, they were observed using the FV31SW fluorescence microscope (Olympus, Tokyo, Japan).

**Statistical Analysis**

Mean ± standard deviation (SD) was used to express the data obtained from no less than 3 autonomous experiments, which were analyzed with Prism 9.0 (GraphPad Software). Statistical analyses included one-way ANOVA and t-test, wherein a p value of less than 0.05 denoted a significant discrepancy.

**RESULTS**

**Lactoferrin Relieved the Acute Alcohol-Induced Liver Injury**

To investigate the impact of lactoferrin on the liver of mice, the mice were given oral doses of lactoferrin at 100 mg/kg and 200 mg/kg for a period of 4 weeks. Following the 4-week period, acute alcoholic liver injury was induced in the mice by administering alcohol (4.8 g/kg bw) through intragastric administration every 12 h for a total of 3 times (Figure 1A). To confirm whether the model was successfully established, the liver injury indicators and histopathological analysis were conducted for detection purposes. As anticipated, there was a noticeable elevation in the liver-to-body weight ratio within the model group in comparison to the control group (Figure 1B). Meanwhile, we observed significant elevations in serum ALT and AST levels in the model group. Additionally, to assess the extent of liver damage, we conducted liver tissue imaging and performed H&E staining. The results of H&E staining revealed the presence of severe lesions in mice after acute alcohol exposure, including irregular hepatic cords, vacuolation of cells, lipid accumulation and aberrant nuclei (Figure 1E). However, the negative alterations were improved following lactoferrin therapy.

**Protective Effect of Lactoferrin on Acute Alcohol-Induced Ferroptosis in Mice Liver**

Alcohol exposure has been shown to significantly induce ferroptosis in hepatocytes (Zhao et al., 2021). In this study, we explored the protective effect of lactoferrin against acute alcohol exposure ferroptosis in mice. Glutathione (GSH) is mainly involved in intracellular scavenging free radicals and maintaining redox homeostasis, and malondialdehyde (MDA) is a prevalent by-product of lipid peroxidation. Both of them are considered critical markers of ferroptosis. As expected, GSH levels were significantly reduced, and MDA levels increased in the model group compared with the control group, whereas lactoferrin improved these changes (Figure 2A-B). We also examined the iron levels in mice liver and demonstrated that treatment with lactoferrin alleviated acute alcohol-induced iron overload (Figure 2C). Finally, the expression levels of the ferroptosis marker proteins ACSL4 and GPX4 were examined. The experimental results presented in Figure 2D indicate a noticeable decrease in GPX4 protein expression and an increase in ACSL4 protein expression upon exposure to acute alcohol. However, lactoferrin treatment partially restored protein expression levels. In conclusion, these results suggested that lactoferrin treatment had the potential to be effective in acute alcohol-induced liver injury by inhibiting ferroptosis.

**Protective Effects of Lactoferrin on Acute Alcohol-Induced Ferroptosis in AML12 Cells**

AML12 cells were treated with 0–100 μg/mL of lactoferrin for 24 h. It was observed that the cell viability started to show a significantly decreasing trend at 100 μg/mL of lactoferrin treatment (Figure 3A). Meanwhile, to investigate the protective effect of lactoferrin against acute alcohol-induced cell death, AML12 cells were treated with different concentrations of lactoferrin for 2 h, followed by treatment with alcohol (300 mM) for 22 h. Finally, we selected 10 μg/mL, 20 μg/mL and 40 μg/mL of lactoferrin to treat AML12 cells based on cell viability (Figure 3B). Lactoferrin pretreatment significantly alleviated the acute alcohol-induced abnormalities of iron overload, elevated MDA, and reduced GSH (Figure 3C-E). Meanwhile, lactoferrin reversed the abnormal expression of GPX4 and ACSL4 induced by alcohol (Figure 3F). Moreover, the excessive production of ROS, lipid ROS and the reduc-
tion of MMP due to alcohol intake were significantly eliminated after lactoferrin administration (Figure 3G-H). In conclusion, the results suggest that lactoferrin protects against acute alcohol-induced liver injury by attenuating ferroptosis.

**Assessment of Iron Chelating Efficacy of Lactoferrin Using AML12 Cells**

To explore the iron chelating impact of lactoferrin, a preliminary experiment involved treating cells with 100 μM FeCl₃ for a duration of 3 h, a condition known to promote cellular iron buildup (Feng et al., 2022). Apart from assessing intracellular iron levels, the extent of iron accumulation was also evaluated through Prussian blue staining and FerroOrange staining methods. Intracellular iron content was significantly downgraded and iron accumulation was significantly alleviated after lactoferrin treatment (Figure 4B-D). Ferritin heavy chain (FTH) plays a vital role in storing iron and is widely acknowledged as a crucial protein for ensuring the balance of iron within the body and maintaining optimal physiological functions. Elevated expression of FTH protein indicates an increase in intracellular iron.

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**Figure 1.** Lactoferrin Relieved the Acute Alcohol-Induced Liver Injury. Scale bar = 100 μm. (A) Animal experimental procedure. (B) Liver/body weight of mice. (C-D) ALT and AST content were measured by kits. (E) Appearance of liver and HE staining. Data are presented as means ± SD. Asterisk (*) vs Control; pound sign (#) vs Ethanol. **P < 0.01, #P < 0.05, and ##P < 0.01.
content and iron loading. Therefore, the expression level of FTH protein was significantly reduced in lactoferrin-treated AML12 cells (Figure 4A). These results above indicated that lactoferrin treatment was consistent with the effect after deferoxamine (DFO) treatment. Based on these results, we concluded that lactoferrin was effective in chelating excess iron in AML12 cells.

**Lactoferrin Alleviated Acute Alcohol-Induced Ferroptosis in Mice Liver by Modulating Iron Metabolism**

To gain more insight into how lactoferrin helps with reducing alcohol-induced iron overload in liver cells of mice, we examined the alterations in the levels of iron regulatory proteins. These proteins include transferrin receptor (TFR, iron input), ferroportin (FPN, iron output), divalent metal transporter 1 (DMT1, iron transport), and ferritin heavy chain (FTH, iron storage). We sought to understand the impact of lactoferrin on the expression of these proteins and their potential role in mitigating iron overload caused by alcohol consumption. After acute alcohol exposure, the mouse liver exhibited significant upregulation of TFR and DMT1, whereas FPN and FTH showed downregulation. This indicates that the overload of intracellular Fe²⁺ could potentially result from intensified iron input alongside reduced iron export and storage (Figure 5A-B).

TFR and DMT1 were significantly decreased, while FPN and FTH were increased in the mouse liver after lactoferrin treatment, indicating that lactoferrin treatment modulates iron metabolism and restores it to homeostasis. Iron content was reflected by Prussian blue staining and lactoferrin reduced intracellular iron accumulation in mice liver (Figure 5C). Collectively, these results suggest that lactoferrin ameliorates acute alcohol-induced iron overload in mice liver by modulating iron metabolism.

**Lactoferrin Alleviated Acute Alcohol-Induced Ferroptosis in AML12 Cells by Modulating Iron Metabolism**

We included a common iron chelator, DFO, for analogy with lactoferrin. Consistent with previous findings, both lactoferrin and DFO modulate the expression of ferroptosis marker proteins GPX4 and ACSL4 and alleviate ferroptosis (Figure 6A). To further determine whether lactoferrin treatment could ameliorate AML12 cells from acute alcohol-induced iron overload, we analyzed changes in iron metabolism-related proteins in AML12 cells. The results were consistent with in vivo

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**Figure 2.** Protective Effect of Lactoferrin on Acute Alcohol-Induced Ferroptosis in Mice Liver. (A and B) GSH and MDA content were measured by kits. (C) Liver iron ion concentration. (D) Expression levels and corresponding statistical analysis of GPX4 and ACSL4 in mice liver. Data are presented as means ± SD. Asterisk (*) vs Control; pound sign (#) vs Ethanol. **P < 0.01 and ##P < 0.01.
Figure 3. Protective Effects of Lactoferrin on Acute Alcohol-Induced Ferroptosis in AML12 Cells. Scale bar = 100 μm. (A-B) Cell viability was determined by MTT assay. (C-E) Content of MDA, GSH, and iron in AML12 cells. (F) Expression levels and corresponding statistical analysis of GPX4 and ACSL4 in AML12 cells. (G) Fluorescence image of MMP. (H) ROS assay kit was used to detect ROS and BODIPY TM 581/591 C11 was used to detect lipid ROS. Data are presented as means ± SD. Asterisk (*) vs Control; pound sign (#) vs Ethanol. * P < 0.05, ** P < 0.01, # P < 0.05, and ## P < 0.01.
results showing TFR and DMT1 significantly upregulated and FPN and FTH downregulated after alcohol treatment (Figure 6B). Meanwhile, FerroOrange and Prussian blue staining were used to reflect intracellular iron content, demonstrating that lactoferrin decreased iron accumulation in acute alcohol-treated cells (Figure 6C-D). In conclusion, lactoferrin ameliorates acute alcohol-induced iron overload in AML12 cells by modulating iron metabolism.

**DISCUSSION**

The multifaceted development of AALI revealed that alcohol-induced liver injury could be reversed by the Fer-1, a ferroptosis inhibitor. This finding highlights the significant role of ferroptosis in the damage caused by alcohol on the liver (Luo et al., 2023; Shi et al., 2023). Therefore, targeting ferroptosis treatment could offer a novel approach to protecting against liver injury induced by alcohol. Many reports have focused on lactoferrin, which has been shown to be hepatoprotective and has a wide range of biological activities (Yin et al., 2010b; a; Xiong et al., 2018). However, most relevant studies have focused on immune regulation, inflammation and oxidative stress, and the mechanism of lactoferrin in relation to ferroptosis was not precisely known. In contrast to previous studies, we aim to investigate the inhibitory effect of lactoferrin on AALI-induced ferroptosis and further validate the therapeutic potential of lactoferrin in modulating iron metabolism to alleviate iron overload. By the study, we found that lactoferrin significantly prevented acute alcohol-induced liver injury by attenuating ferroptosis in mice liver and AML12 cells. Mechanistically, our research showed that lactoferrin inhibited ferroptosis by iron chelation and regulating iron metabolism, thus revealing the preventive or therapeutic potential of lactoferrin in acute alcohol-induced liver injury.

In this study, an acute alcohol gavage protocol was used to establish a suitable experimental model of

![Figure 4.](image-url)
AALI by short-term multiple alcohol ingestion. The liver weight/body weight ratio, AST, and ALT levels of mice in the model group were significantly elevated compared with the control group, while pathological changes in the livers of the mice could be observed by tissue image analysis. The results showed that our animal model of AALI was reliable. Meanwhile, the study provided evidence for the beneficial effects of lactoferrin in reducing acute alcohol-induced liver injury. These results are generally consistent with previous studies (Li et al., 2021, 2022a). Alcohol-induced liver injury is associated with iron overload and ferroptosis induction (Liu et al., 2020a; Zhao et al., 2021). Therefore, we initiated a study targeting acute alcohol-induced ferroptosis. Excessive accumulation of reactive oxygen species (ROS), lipid peroxides and intracellular iron is the critical feature of ferroptosis (Yu et al., 2020; Chen et al., 2022). We examined relevant biochemical indices in vivo and in vitro. Overall, we found that indicators related to acute alcohol-induced ferroptosis were alleviated by lactoferrin treatment.

To explore the specific mechanisms by which lactoferrin alleviates ferroptosis, we hypothesized that lactoferrin mitigated intracellular iron overload through its iron chelating capacity. As we all know, an excess of iron is harmful because oxidative stress can cause cell damage and even lead to iron-dependent cell death, a condition known as ferroptosis (Zheng et al., 2021). Therapeutic intervention with iron chelators is an important strategy for removing excess iron. Previous investigations have illustrated that lactoferrin obstructed the interaction between superoxide molecules and free iron (Fe²⁺) ions through iron chelation. This boundary condition hindered the creation of iron (Fe²⁺) salts and basal oxygen. In turn, the iron (Fe²⁺) ions involved in the Fenton reaction were less reactive (Kruzel et al., 2017). On this basis, we explored the effect of the iron-chelating properties of lactoferrin on iron overload in the present study. First, we needed to verify whether lactoferrin could effectively chelate excess iron in AML12 cells. Our results revealed that iron content in AML12 cells was significantly elevated after FeCl₃ treatment and decreased after lactoferrin treatment.
Figure 6. Lactoferrin Alleviated Acute Alcohol-Induced Ferroptosis in AML12 Cells by Modulating Iron Metabolism. Scale bar = 100 μm. (A) Expression levels and corresponding statistical analysis of GPX4 and ACSL4 in AML12 cells. (B) Expression levels and corresponding statistical analysis of FTH, FPN, TFR, and DMT1 in AML12 cells. (C) Image of FerroOrange staining of AML12 cells. (D) Image of Prussian blue staining of AML12 cells. Data are presented as means ± SD. Asterisk (*) vs Control; pound sign (#) vs Ethanol. **$P < 0.01$, # $P < 0.05$, and ## $P < 0.01$. 

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treatment. Deferoxamine (DFO) is a clinical treatment that effectively reduces iron availability in the body by directly chelating iron (Chen et al., 2020). Through the process of endocytosis, DFO enters the cell where it forms a stable compound called ferrous amine with Fe$^{3+}$. This interaction effectively reduces the levels of unstable iron within the cell. By utilizing DFO as a treatment, the availability of iron is significantly decreased in a controlled and targeted manner (Leu et al., 2019). Therefore, we added DFO to our experiments and found that lactoferrin had the same effect as DFO. The result indicated that lactoferrin could efficiently chelate intracellular iron in AML12 cells.

In addition to iron chelating properties, we speculated that lactoferrin also alleviated ferroptosis through other pathways. It has been shown that DFO attenuated ionizing radiation (IR) induced ferroptosis and intestinal damage in vitro and in vivo (Zhou et al., 2022). Meanwhile, DFO regulated iron metabolism and alleviated intracellular iron overload by restoring abnormalities in iron metabolism (Zeng et al., 2021; Feng et al., 2023). Iron metabolism is the sensitive control of extracellular and intracellular iron dynamics. The cells need to maintain a balance in their intracellular iron content to support metabolism while preventing excessive iron accumulation that could be toxic to the cell. This delicate equilibrium is essential for cellular functioning and survival. The process is coordinately regulated by a network of proteins for the absorption, storage, recycling and utilization of iron (Drakesmith et al., 2015; Du et al., 2019; Liu et al., 2020c). In this investigation, the analysis focused on 4 proteins associated with iron metabolism: Transferrin Receptor (TFR), Divalent Metal Transporter 1 (DMT1), Ferritin Heavy Chain (FTH), and Ferroportin (FPN). The aberrant expression of these proteins led to increased iron uptake, decreased iron storage, and restricted iron efflux. We found that all 3 of these states occurred after acute alcohol treatment, as evidenced by increased expression of TFR, DMT1, and decreased expression of FTH, FPN. The accumulation of intracellular iron resulting from this atypical condition impacts cell sensitivity toward ferroptosis (Xie et al., 2016; Fang et al., 2023).

As mentioned previously, both DFO and lactoferrin were iron chelating components, therefore we hypothesized that lactoferrin also had the ability to regulate iron metabolism. Iron metabolism can be regulated through 3 primary mechanisms: managing iron intake (Sun et al., 2015), synthesizing diverse iron-containing proteins derived from the unstable iron state within the iron pool (Sato et al., 2018), and facilitating the transportation of iron out of the cell (Brown et al., 2019). Fe$^{3+}$ from the extracellular environment forms a bond with TF and is subsequently internalized into the endosomes via its receptor TFR. The iron ion, Fe$^{3+}$, undergoes reduction to Fe$^{2+}$ courtesy of the Six-Transmembrane Epithelial Antigen of Prostate 3 (STEAP3) protein, after which it is conveyed to the cytoplasm through DMT1 (Torti and Torti, 2013). Fe$^{2+}$ entering the cytoplasm preferentially forms a variety of iron-binding complexes, with ferritin being the major iron storage protein, consisting of FTL and FTH (Arosio and Levi, 2010). When the levels of these complexes approach saturation, excess iron can be exported from the cell via FPN on the cell membrane (Anderson and Frazer, 2017). In our study, the expression of proteins related to iron metabolism was likewise determined. We found that acute alcohol treatment resulted in excess divalent iron in labile iron pool (LIP). Loss of cell function and ferroptosis occur due to the exacerbation of the fenton reaction by the divalent iron in LIP. This exacerbation leads to the generation of reactive oxygen species (ROS) substances, specifically hydroxyl radicals. The accumulation of these ROS substances further causes peroxidation of membrane lipids, ultimately resulting in the impairment of cell function. In contrast, the expression of all iron metabolism-related proteins was restored and intracellular iron overloading was alleviated after lactoferrin treatment, which was consistent with the effect of DFO. Consequently, our results confirmed that lactoferrin attenuated intracellular iron overload by modulating iron metabolism.

Lactoferrin possesses a remarkable affinity for binding iron and plays a crucial role in maintaining cellular iron homeostasis by effectively storing and releasing iron (Shini et al., 2022). However, our results found that lactoferrin could not completely inhibit iron overload and eliminate ferroptosis, so we hypothesized that other mechanisms may exist to regulate iron metabolism. Relevant studies have confirmed that lactoferrin has the ability to inhibit excessive autophagy (Pan et al., 2013; Wu et al., 2022). It has been observed that alcohol treatment leads to increased autophagy in hepatocytes and worsens nuclear receptor coactivator-4 (NCOA4)-mediated autophagic degradation of ferritin (Zhao et al., 2021; Liu et al., 2022; Song et al., 2022). This is evident from the rise in free intracellular iron ions due to NCOA4 carrying FTH to the lysosome and releasing iron from ferritin into the LIP (Fuhrmann et al., 2020; Zhou et al., 2022). Based on this, we hypothesized that regulation of autophagy may be a key underlying mechanism of lactoferrin in the regulation of iron metabolism. Consequently, we hypothesized that lactoferrin reduced the levels of free iron ions by inhibiting ferritin autophagy, which in turn lead to the upregulation of FTH and reduction of free iron ions. In addition to autophagy, relevant studies have demon-
strated that the protective effects of lactoferrin are mediated by activation of Nrf2 (Hu et al., 2020, 2023; Mohamed et al., 2023). In light of our prior investigations, it has been indicated that the pathway involving p62/Keap1/Nrf2 is associated with the control of ferroptosis and iron metabolism (Zhao et al., 2021). In particular, restraint of autophagy results in heightened build-up of p62. Furthermore, p62 interacts with Keap1, which is akin to Kelch-like ECH-associated protein 1, enabling the movement of Nrf2 into the nucleus and consequent increase in FTH and FPN. This complex mechanism ultimately aids in the regulation of iron metabolism. Furthermore, it has been demonstrated that hepcidin, a crucial regulator of iron metabolism, is downregulated in cell lines and animal models after alcohol treatment (Bridle et al., 2006; Harrison-Findik et al., 2007). This downregulation of hepcidin leads to an elevated duodenal iron transport and uptake in animals, consequently causing iron overload (Harrison-Findik et al., 2006). It has been demonstrated that lactoferrin can alleviate hepatocellular iron overload caused by non-alcoholic fatty liver disease (NAFLD) by targeting hepcidin (Guo et al., 2020). Therefore, we speculated that lactoferrin can also regulate hepcidin abnormalities caused by AALI. These potential mechanisms mentioned above are the direction of our future research, and we will focus on autophagy, Nrf2-associated pathways, and hepcidin to further elucidate the mechanism of action of lactoferrin.

In conclusion, the present study proposed that lactoferrin alleviated acute alcohol-induced ferroptosis in mice liver and AML12 cells through iron chelation and regulation of iron metabolism. This study reveals that lactoferrin may mitigate the adverse effects of acute alcohol on liver injury by attenuating ferroptosis and holds promise as a potential dietary modifier against AALI.

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