JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Lactoferrin Alleviates Acute Alcoholic Liver Injury by Improving Redox-Stress Response Capacity in Female C57BL/6J Mice

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Cite This: https://doi.org/10.1021/acs.jafc.1c06813 Read Online

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ABSTRACT: Lactoferrin (Lf) can attenuate alcoholic liver injury (ALI) in male mice; however, the effects of Lf on acute ALI in female mice are still unknown. Female C57BL/6J mice were randomly divided into four groups and fed with different diets for 4 weeks: an AIN-93G diet for control (CON) and ethanol (EtOH) groups; an AIN-93G diet with 0.4 and 4% casein replaced by Lf for low-dose Lf (LLf) and high-dose Lf (HLf) groups. Acute ALI was induced by intragastric administration of ethanol (4.8 g/kgbw) every 12 h continuously for three times. HLf had obvious alleviating effects on acute ALI. Lf pretreatment did not affect hepatic alcohol metabolism key enzymes. Meanwhile, the ethanol-induced hepatic reactive oxygen species level increase was not ameliorated by Lf. Metabolomics and bioinformatics analysis results suggested an important role of redox-stress response capacity (RRC). Western blots showed HLf-promoted AKT and AMP-activated protein kinase activations and upregulated Nrf2 and LC3-II expressions, which was associated with RRC improvement. In summary, HLf could prevent acute ALI in female mice, and RRC likely played an important role.

KEYWORDS: *lactoferrin, acute alcoholic liver injury, female, redox-stress response capacity*

INTRODUCTION

Alcohol-associated liver disease (AALD) is a serious public health problem with a relatively high prevalence.[1] Globally, over 50% of mortality related to cirrhosis is attributable to alcohol.[1] The annualized age-standardized deaths due to AALD are projected to nearly double (from 8 to 15 per 100,000 person-years) by 2040 if left without strong intervention.[2] Due to the absence of efficient pharmacological treatment at present, the AALD prevention by natural compounds has raised research attention.[3,4]

Lactoferrin (Lf) is an iron-binding protein possessing various beneficial biological activities,[5,6] and decreased hepatic Lf is associated with hepatic steatosis in nonalcoholic fatty liver disease (NAFLD).[7] Some studies have reported that Lf exhibits fair application potentials in NAFLD.[8−10] Since AALD has an overlapping pathophysiology with NAFLD,[11] it is likely that Lf has protective effects on AALD. Moreover, our previous study also demonstrated the preventive effects of Lf on ethanol-induced liver injury in male mice.[12] However, this experimental model simultaneously mimicked acute and chronic alcoholic liver injury (ALI). Acute ALI and chronic ALI do not share the common pathogenesis.[13] Additionally, there are gender differences in alcohol sensitivity.[14] In general, due to the ethanol metabolism capacity difference and the hormone influence, alcohol susceptibility in male is far lower than that in female, especially after an acute large dose of alcohol intake.[14] Thus, we speculated that the preventive effects and mechanisms of Lf on ALI possibly vary with alcohol exposure patterns and genders.

In the pathogenesis of ALI, reactive oxygen species (ROS) and redox homeostasis abnormity play a crucial role.[15,16] The alcohol metabolism process can generate massive ROS and exert adverse biological effects, especially with the catalysis of cytochrome P450 2E1 (CYP2E1).[17,18] Some researchers think suppressing hepatic ROS production and alleviating oxidative stress are a viable strategy for AALD prevention and treatment, which have been supported by substantial experimental evidence.[19−21] However, ROS also has positive effects on normal physiological process maintenance,[22] and moderate rather than low ROS generation is more beneficial to health.[23] A recent study proposed a new concept "redox-stress response capacity (RRC)", which refers to cells or organisms that are able to generate dynamic redox responses to activate cellular signaling and maintain cellular homeostasis.[23] The theory emphasized that dynamic RRC may be more important than the simple ROS level in redox balance regulation.[23] Considering the closed association between ROS and ALI, the effects of RRC on ALI pathogenesis deserved our exploration.

Sexual dimorphism is currently rarely considered in the prevention and treatment of liver disease.[24] Nevertheless, evidence shows that women until the 1980s birth cohort have been increasingly likely to become binge drinkers, especially in some developed countries.[25] Therefore, in this study, we aimed to investigate the preventive effects of Lf on acute ethanol intake-induced liver injury in female mice and examine whether the similar mechanisms in male mice are also involved in the regulatory process in female mice. In addition, other potential mechanisms also were further explored in the study.

Received: October 27, 2021
Revised: December 1, 2021
Accepted: December 1, 2021

MATERIALS AND METHODS

Reagents. Native bovine lactoferrin was obtained from Hilmar Cheese Co, Inc. (CA, USA). Anhydrous ethanol (guaranteed reagent) was obtained from Chinasun Specialty Products Co, Ltd. (Jiangsu, China).

Animals and Treatments. A total of 38 mice (6−8 weeks old) were purchased from Shanghai Jihui Laboratory Animal Care Company and housed in a standard SPF animal laboratory (temperature 20−26 °C; relative humidity 40−60%; 12 h light−dark cycle) with access to food and liquid ad libitum. After 1 week of acclimation, they were randomly divided into four groups: (1) control group (CON, n = 8), (2) acute alcoholic liver injury model group (EtOH, n = 10), (3) low-dose Lf group (LLf, n = 10), and (4) high-dose Lf group (HLf, n = 10). Mice were fed with a AIN-93G diet in CON and EtOH groups, whereas AIN-93G diets with 0.4 and 4% casein replaced by Lf were used in the LLf group and the HLf group, respectively. The diet ingredients are displayed in Table 1. The basis of the Lf dose settings has been demonstrated in the previous study.[12] After feeding of 4 weeks, acute ALI was induced by intragastric administration of ethanol (4.8 g/kgbw) every 12 h for a continuous three times (Figure 1A). The food and liquid consumptions were recorded once a week.

Table 1. Diet Ingredients[a]

ingredient (g)	CON	EtOH	LLf	HLf
casein	200	200	199.2	192
lactoferrin	0	0	0.8	8
L-cystine	3	3	3	3
sucrose	100	100	100	100
corn starch	397.5	397.5	397.5	397.5
maltodextrin	132	132	132	132
soybean oil	70	70	70	70
cellulose	50	50	50	50
mineral mix #210025	35	35	35	35
vitamin mix #310025	10	10	10	10
choline bitartrate	2.5	2.5	2.5	2.5
total	1000	1000	1000	1000
kcal/g	4	4	4	4

[a]CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group.

All procedures followed the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The animal experiments were approved by the Soochow University Animal Ethics Committee (approval no. 202009A661).

Sample Collection. All mice were sacrificed under anesthesia by pentobarbital after ethanol exposure and fasting overnight. The serum samples were prepared from blood by centrifugation at 3000 rpm for 10 min and stored. The liver samples were immediately harvested, weighed, frozen by liquid nitrogen, and stored for further analyses.

Serum Transaminase Level Determination. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using commercial kits (Solarbio, Beijing, China) according to the manufacturer's protocols.

Hepatic Enzyme Activity Determination. Hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities were determined using the corresponding assay kits (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Hepatic superoxide dismutase (SOD) levels and glutathione peroxidase (GPx) levels were determined using a total SOD assay kit and a GPx assay kit (Beyotime, Shanghai, China), respectively. Hepatic catalase (CAT) activity was tested using a commercial kit (Solarbio, Beijing, China).

Histological Analysis for the Liver. Liver tissues fixed in 4% paraformaldehyde were sent to Servicebio (Wuhan, China) for embedding, sectioning, and staining with hematoxylin and eosin (HE). In addition, the sections were also stained with Perls for hepatic iron accumulation assessment. The images were observed and acquired using a Leica DMI8 digital microscope (Wetzlar, Germany).

Hepatic ROS Level Determination. ROS level determination, by its gold standard electron spin resonance (ESR),[26] was performed as previously described.[12] The liver samples were homogenized with DMSO (Beyotime, Shanghai, China). The supernatants were collected by centrifuging at 3500 × g for 5 min at 4 °C. The supernatants were mixed with DMPO (Dojindo, Shanghai, China) in a volume ratio of 10:1. Then, the mixture was loaded in a 50 μL glass capillary tube and detected immediately. ESR spectra were recorded using a Bruker ELEXSYS-II E500 spectrometer (Karlsruhe, Germany) at room temperature. Typical spectrometer settings are listed as follows: magnetic field center 3504 G, sweep width 100 G, microwave frequency 9.84 GHz, microwave power 20 mW, time constant 0 s, sweep time 20 s, and scan number 3.

Hepatic Targeted Metabolomics. Approximately 30 metabolites in the process of tricarboxylic acid cycle, glycolysis pathway, oxidative phosphorylation, and pentose phosphate pathway in the liver were quantified via multiple reaction monitoring. This determination was performed by Bioprofile Technology Company (Shanghai, China), and the predicted differences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways were further analyzed. The detailed experimental protocols are given in Supplementary File 1.

Western Blots. Western blots were performed as previously described.[27] Briefly, hepatic total protein was extracted using RIPA lysis buffer containing a protease inhibitor (Beyotime, Shanghai, China). Then, the protein samples were mixed with 5× dual color protein loading buffer (Fudebio, Hangzhou, China), and boiled at 98 °C for 10 min. Equal amounts of protein (30 μg/lane) were loaded on an SDS-PAGE gel at a constant voltage, then the gels were transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). After blocking, incubating with the primary antibody, and incubating with the secondary antibody, the bands were visualized using a chemiluminescence image analysis system (Tanon, Shanghai, China) with FDbio-Femto ECL (Fudebio, Hangzhou, China). The band intensities were quantified using Gel-Pro Analyzer software (Media Cybernetics, Maryland, USA). Antibody information is shown in Table 2.

Statistical Analyses. All data were presented as "mean ± standard deviation (SD)." One-way ANOVA followed by the LSD post hoc test were used to analyze differences among groups. $P < 0.05$ was considered as statistical significance. All analyses were performed using SPSS version 21.0 (IBM, New York, USA).

RESULTS

Basic Profiles of the Mice. All mice survived during the experiment. As shown in Figure 1B, Lf did not influence the food consumptions of the mice. Naturally, there was no obvious difference in the body weights among the four groups during the feeding period; however, a body weight reduction happened in all mice before sacrifice due to fasting overnight, and the loss was more obvious in alcohol-treated mice (Figure 1C).

Effects of Lf on Liver Weights and Serum Transaminase Levels. EtOH intervention significantly increased the liver weight and hepatic triglyceride content, whereas the two parameters were significantly reduced by HLf pretreatment (Figure 1D,E). Lf could dose-dependently restrain the alcohol-induced serum ALT and AST elevations, but the decreases were significant only in the HLf group and not the LLf group (Figure 1F).

Effects of Lf on Hepatic Histological Changes. Acute alcohol administration induced obvious histological changes in the liver, mainly manifested as macrovesicular steatosis, diffuse microvesicular steatosis, a few inflammation responses, and severe ballooning. Lf pretreatment could ameliorate the hepatic morphological structure, and the effects were more pronounced in the HLf group than the LLf group. In the LLf group, hepatic histological changes mainly were microvesicular steatosis and a

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

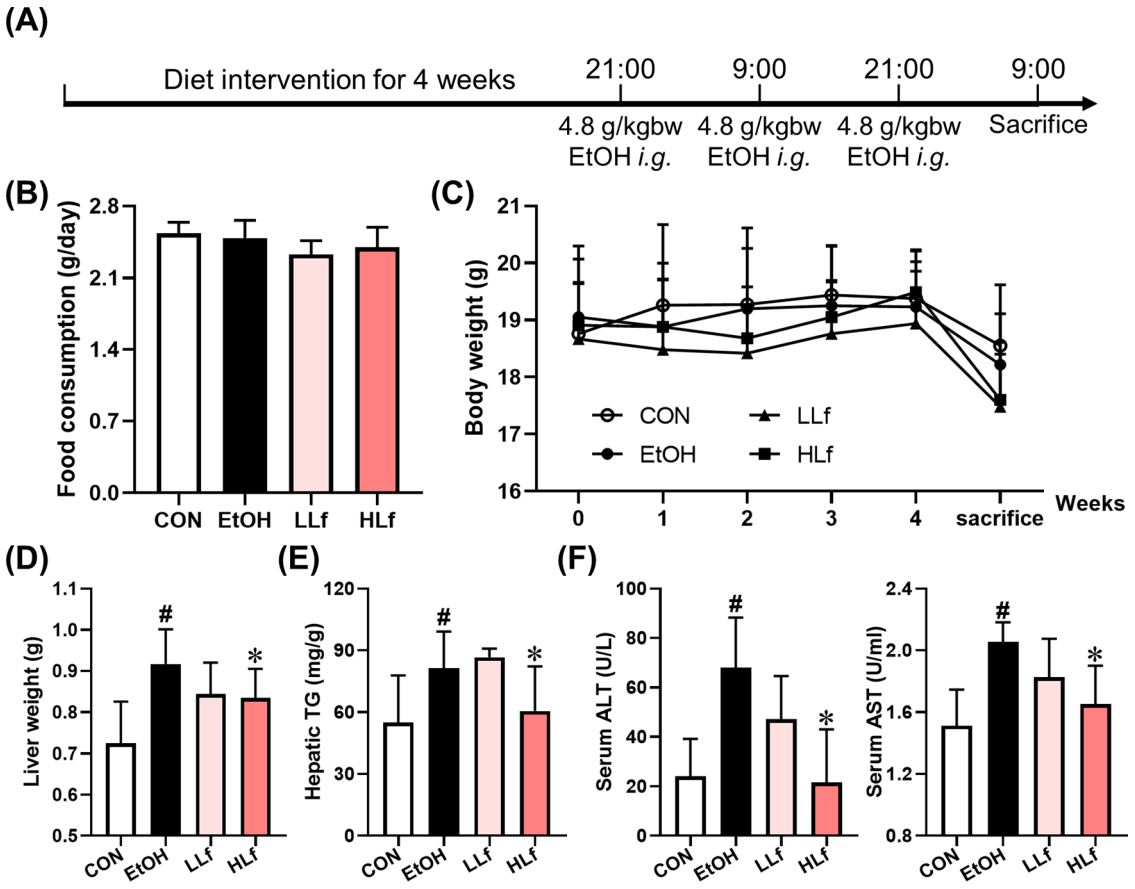

Figure 1. Experimental design and basic indices of the mice. (A) Experimental design. (B) Food consumptions of the mice. (C) Changes of body weights. (D) Effects of Lf on the liver weight. (E) Effects of Lf on hepatic triglyceride content. (F) Effects of Lf on serum transaminase levels. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N = 8$, for the CON group; $N = 10$, for EtOH, LLf, and HLf groups. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

little macrovesicular steatosis, and the morbid hepatic morphological structure was furtherly attenuated in the HLf group (Figure 2A). Meanwhile, almost no Perls positive reaction was observed, namely, neither alcohol intervention nor HLf pretreatment led to obvious hepatic iron accumulation (Figure 2B).

Effects of Lf on Hepatic Alcohol Metabolism. As displayed in Figure 3A,B, an overexpression of hepatic CYP2E1 protein was observed after alcohol administration, while Lf pretreatment did not exert a significant effect on CYP2E1 expression. Alcohol intervention upregulated ALDH activity but not ADH activity; however, neither ALDH activity nor ADH activity in the liver was affected by Lf pretreatment (Figure 3C).

Effects of Lf on Hepatic ROS Levels. All liver samples exhibited the characteristic ESR spectra of superoxide and ascorbic acid radicals, and the signal intensities seemed to be more intense in mice with ethanol exposure than those without exposure (Figure 4A). Meanwhile, no meaningful ESR signals were captured in the blank control (Figure 4B). Quantitative analyses are shown in Figure 4C; alcohol intervention elicited hepatic ascorbate free radicals and superoxide level elevation. Although Lf tended to decrease ascorbate free radical levels, the differences were not significant. Lf failed to reduce hepatic superoxide levels, even its level was higher in the LLf group than the EtOH group.

Effects of Lf on Hepatic Metabolites. As shown in Figure 5, a distinct hepatic metabolite relative abundance was exhibited between the mice with and without Lf pretreatment. Compared with the group without Lf pretreatment, hepatic 6-phosphogluconate, L-malate, L-glutamate, cAMP, AMP, and flavin mononucleotide (FMN) levels were obviously higher, while the D-fructose-6-phosphate, D-glucose-1-phosphate, D-glucose-5-phosphate, and guanosine diphosphate (GDP) levels were lower. Furthermore, we performed KEGG analysis to screen for the potential signaling pathways involved in the acute ALI preventive process of Lf. The screened pathways or physical processes were as follows: glucagon signaling pathway, citrate cycle, carbon metabolism, glycolysis, oxidative phosphorylation, AMP-activated protein kinase (AMPK) signaling pathway, insulin secretion, and so on (Supplementary File 2).

Effects of Lf on Hepatic Redox-Sensitive Protein Levels. Acute alcohol exposure did not affect total protein kinase B (AKT) protein expression but tended to inhibit AKT phosphorylation. Lf could upregulate AKT protein expression levels, and HLf significantly promotes AKT activation (Figure 6A). Lf had no effects on the alcohol-induced phosphorylation increase of extracellular signal-regulated kinase 1/2 (ERK1/2) (Figure 6B). In addition, Lf upregulated AMPK phosphorylation levels in a dose-dependent manner without affecting AMPK total protein levels (Figure 6C). Correspondingly, a significantly increased ratio of AMP to ATP was observed in Lf-treated groups (Figure 6D).

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Table 2. Antibody Information[a]

antibody	manufacturer	country	dilution
CYP2E1	Abcam	UK	1/5000
AKT	CST	USA	1/1000
P-AKT	CST	USA	1/1000
ERK1/2	CST	USA	1/2000
p-ERK1/2	CST	USA	1/2000
AMPK	ImmunoWay	USA	1/1000
p-AMPK	ImmunoWay	USA	1/1000
CPT1A	Proteintech	CN	1/2000
Nrf2	Proteintech	CN	1/1000
Keap1	Proteintech	CN	1/5000
PI3K	HUABIO	CN	1/1000
LC3	CST	USA	1/1000
p62	CST	USA	1/1000
HSP60	Proteintech	CN	1/1000
HSP90	Proteintech	CN	1/1000
PSMD13	ABclonal	CN	1/1000
PSME1	Abcam	UK	1/3000
GAPDH	Proteintech	CN	1/20000

[a]CYP2E1, cytochrome P450 2E1; AKT, protein kinase B; p-AKT, phospho-AKT; ERK1/2, extracellular signal-regulated kinase 1/2; p-ERK1/2, phospho-ERK1/2; AMPK, AMP-activated protein kinase; p-AMPK, phospho-AMPK; CPT1A, carnitine palmitoyltransferase 1A; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; PI3K, phosphatidylinositol 3-kinase; LC3, microtubule-associated protein light chain 3; p62, sequestosome 1; HSP60, heat shock protein 60; HSP90, heat shock protein 90; PSMD13, proteasome 26S subunit non-ATPase 13; PSME1, proteasome activator subunit 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

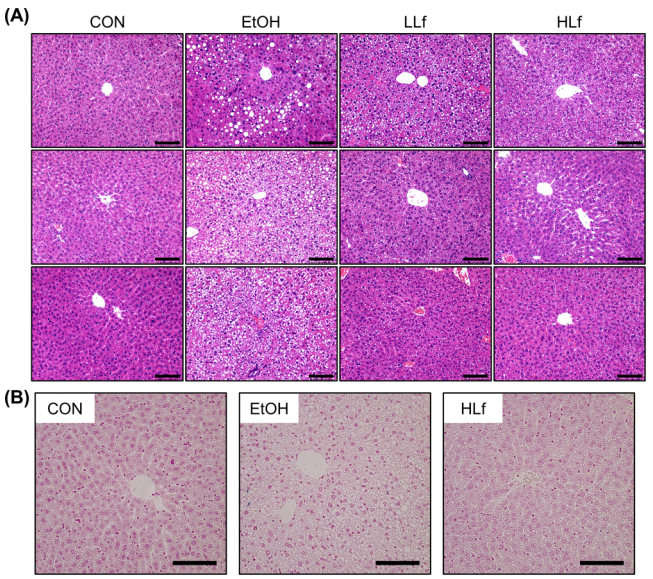

Figure 2. Effects of Lf on hepatic and iron accumulation. (A) Representative images of the livers with HE staining for three mice in each group. (B) Representative images of the livers with Perls staining. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Scale bar, 100 μm.

Effects of Lf on Maintaining Redox Homeostasis Ability in the Liver. As shown in Figure 7A,B, acute alcohol intervention significantly suppressed carnitine palmitoyltransferase 1A (CPT1A) protein expression in the liver, while it was upregulated by Lf pretreatment in a dose-dependent manner, and the difference was significant in the HLf group compared with the EtOH group. Both LLf and HLf pretreatments significantly increased nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression and significantly reduced Kelch-like ECH-associated protein 1 (Keap1) expression at the protein level. Moreover, compared with the EtOH group, an increased phosphatidylinositol 3-kinase (PI3K) protein level was observed only in the HLf group but not in the LLf group.

Hepatic antioxidative enzyme activities are shown in Figure 7C. Alcohol intervention led to GPx and CAT activity elevations. Neither LLf treatment nor HLf treatment changed GPx activity and only HLf could significantly promote hepatic CAT activity. Compared with the CON group, SOD activity was significantly inhibited in the EtOH group, which could be slightly alleviated by Lf pretreatment; however, the differences were insignificant.

Effects of Lf on the Expressions of Protein Related to Degradation of Damaged Proteins. HLf pretreatment had a more potent preventive effect, we therefore focused on the effects of HLf on the expressions of proteins affecting the damaged protein degradation. First, we tested the autophagy-related protein expression levels (Figure 8A). EtOH administration led to a significant reduction in microtubule-associated protein light chain 3-II (LC3-II) expression and a slight increase in sequestosome 1 (p62) protein levels. LC3-II protein levels could be significantly increased by HLf pretreatment; meanwhile, HLf pretreatment exhibited a potential to reduce hepatic p62 levels, although without statistical significance. Second, we detected the levels of chaperones represented by heat shock proteins (HSPs) (Figure 8B). Neither HSP60 nor HSP90 was sharply affected by acute alcohol exposure. Compared with the EtOH group, the protein levels of both HSP60 and HSP90 increased by about 20% in the HLf group, but the differences were insignificant. Third, the effects of Lf on the proteasome system were also investigated (Figure 8C). Although proteasome 26S subunit non-ATPase 13 (PMSD13) (as a proteasome structural subunit) protein levels were consistent among the three groups, alcohol administration significantly suppressed proteasome activator subunit 1 (PSME1) expression at the protein level; meanwhile, HLf pretreatment exhibited few potentials to restore PSME1 protein expression.

DISCUSSION

In this study, we found that HLf pretreatment possesses more potent preventive effects on ALI induced by acute alcohol exposure in female mice, and the potential mechanisms are associated with RRC elevation rather than hepatic alcohol metabolism regulation like in male mice.

Short-time multiple ethanol administration is a common and suitable experimental model for acute ALI research.[28−30] In our present study, compared with the CON group, the liver weights, hepatic triglyceride contents, and serum transaminase levels were significantly higher in the mice of the EtOH group. Meanwhile, histological analysis indicated that the morphological structure of the mouse liver exhibited obvious lesions after acute alcohol exposure. These results indicated that our animal model of acute ALI is convincing. Unlike LLf, which has more obvious preventive effects in male mice,[12] Lf pretreatment reduced the liver weights and serum transaminase levels in a dose-dependent manner and the differences were significant only in the HLf group. The ameliorating effects of HLf were more obvious on hepatic morphology than LLf, which illustrated

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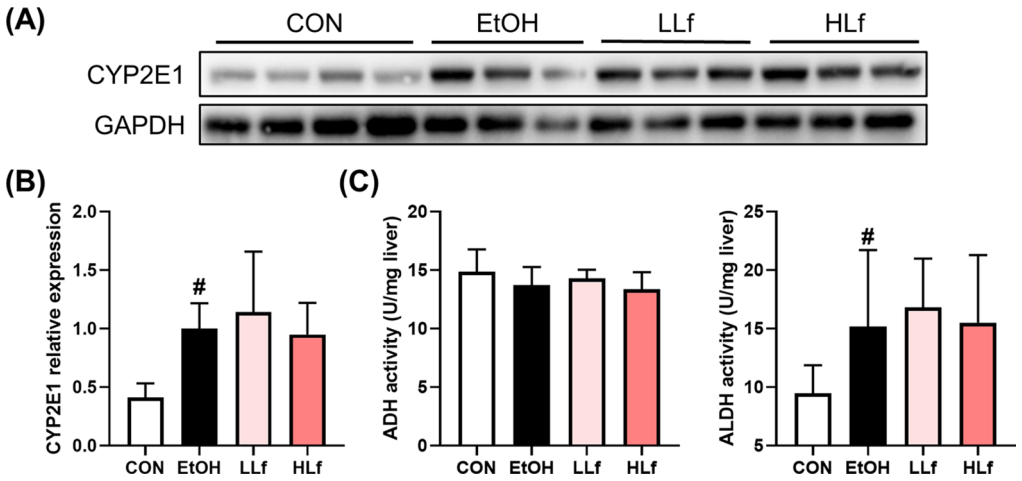

Figure 3. Effects of Lf on hepatic alcohol metabolism key enzymes. (A) Representative western blot image for CYP2E1. (B) Hepatic CYP2E1 protein expression levels. (C) Hepatic alcohol dehydrogenase and aldehyde dehydrogenase activities. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N \geq 6$, for each group. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

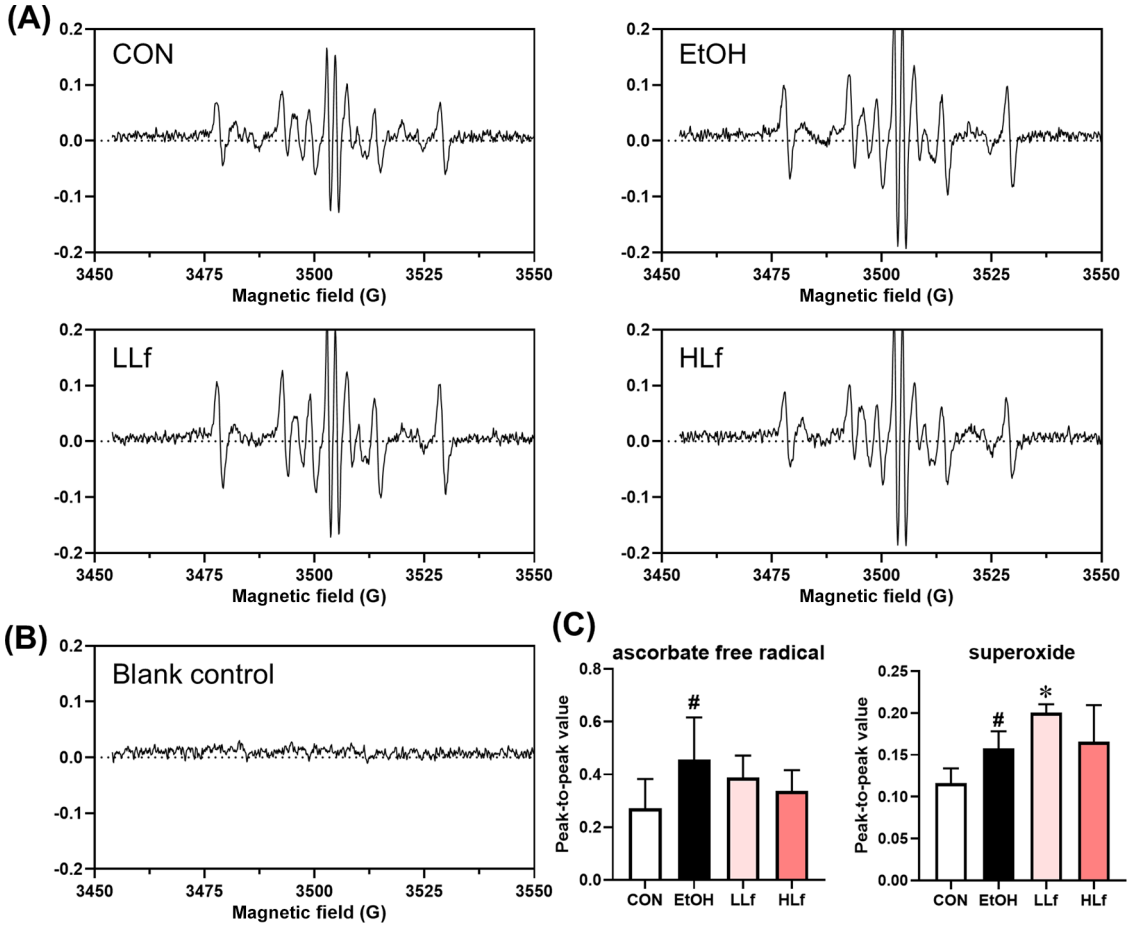

Figure 4. Effects of Lf on hepatic ROS levels. (A) Representative ESR spectra of the hepatic homogenate supernatants of each group. (B) ESR spectrum of the blank control. (C) Quantitative analysis for hepatic superoxide and ascorbic acid radical levels. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N \geq 4$, for each group. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

that HLf pretreatment had more potent preventive effects of acute ALI for the female mice. The findings suggested that gender should be considered for the personalized Lf supplement strategy.

Heavy alcohol consumption is usually regarded as a risk factor for weight gain in humans, although the overall results is not conclusive.[31] Nevertheless, a weight loss was observed in alcohol-treated mice. On the one hand, the positive association between alcohol intake and weight gain is mainly from studies

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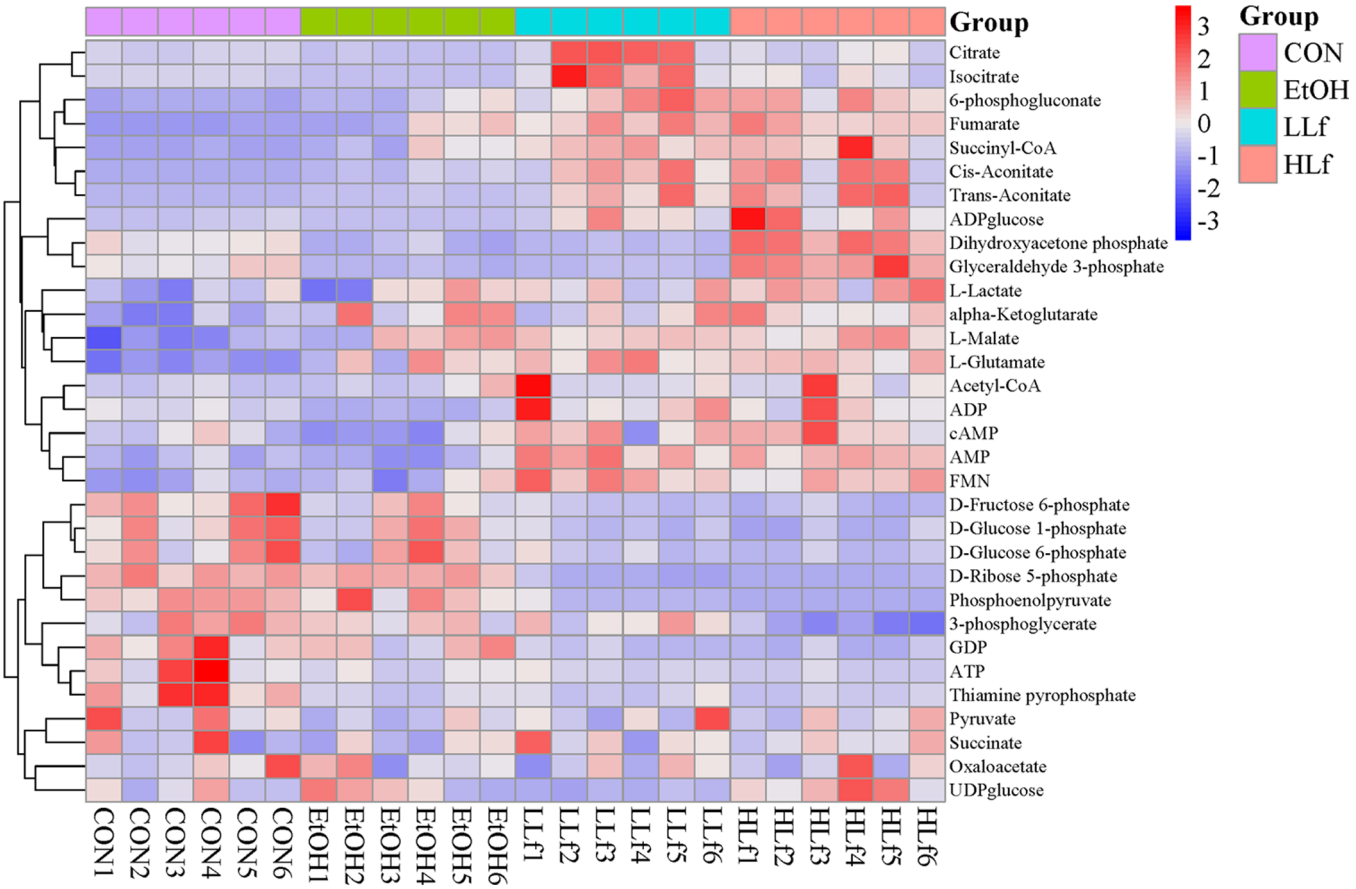

Figure 5. Effects of Lf on the metabolites in the liver. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. $N = 6$ for each group.

on long-term heavy drinkers, which might be not applicable to the acute alcohol administration model. On the other hand, weight loss itself is one of the toxic effects of acute alcoholism, which was also reported by other researchers.[32]

Our previous study found that Lf prevents ALI in male mice mainly by regulating hepatic alcohol metabolism.[12] Thus, we first investigated whether similar regulatory mechanisms were involved in the acute ALI prevention in female mice. As reported by many studies,[33−35] our study also found that acute alcohol intake upregulated CYP2E1 protein expression and increased ALDH activity. However, neither LLf nor HLf affected the alcohol metabolism key enzymes including CYP2E1, ADH, and ALDH. Unlike male mice,[12] Lf did not inhibit the alcohol-induced hepatic ROS level increase in female mice. Although superoxide is not a strong oxidant, it may be one of the most abundant ROS in the body, and it is a precursor of most other ROS.[36] Thus, superoxide can reflect the total ROS level, at least to a certain extent.[12] In our study, no hydroxyl radical and hydrogen peroxide ESR signals were captured, likely due to their relative low content. These suggested that the acute ALI prevention of Lf in female mice did not depend on hepatic alcohol metabolism regulation, and other mechanisms might play a more important role.

Thus, to further explore the potential mechanisms, a targeted metabolomics analysis was performed, and the KEGG metabolic pathways were also analyzed. The results suggested that the process of Lf to prevent acute ALI might be implicated in many pathways, such as the glucagon signaling pathway, citrate cycle, oxidative phosphorylation, and AMPK signaling pathway. Interestingly, these metabolic pathways are directly or indirectly related to redox homeostasis regulation.[37−43] Meanwhile, based on the important role of oxidative stress in alcohol-induced liver injury[15] and the close association between Lf and hepatic ROS levels, we speculated that redox balance regulation played an important role in Lf-mediated acute ALI prevention of female mice.

RRC refers to cells or organisms that are able to generate dynamic redox responses to activate cellular signaling and maintain cellular homeostasis.[23] RRC mainly includes three abilities: the ability to generate ROS, the ability to activate cellular signaling and regulate the antioxidant system, and the ability to degrade damaged proteins.[23] The ESR results confirmed the integrality of the ability to generate ROS in these mice. Thus, we investigated the effects of Lf on redox-sensitive signaling pathways, including AKT, ERK1/2, and AMPK signaling pathways.[23,43−45] These signaling pathways play a critical role in sensing and responding to ROS and oxidative stress.[43−45] In general, their activations are conducive to maintain the organic redox balance. As reported by others,[46] the phosphorylation of ERK1/2 was promoted after alcohol administration, which probably was a positive adaptation for organisms to respond to alcohol-induced stress. Although ERK1/2 was not further activated by Lf pretreatment, both AKT activity and AMPK activity were increased in the HLf treatment group. Moreover, the elevated AMP/ATP ratio in Lf groups also further support AMPK activation.[47,48] Thus, HLf pretreatment could enhance the ability to activate redox-sensitive signaling pathways.

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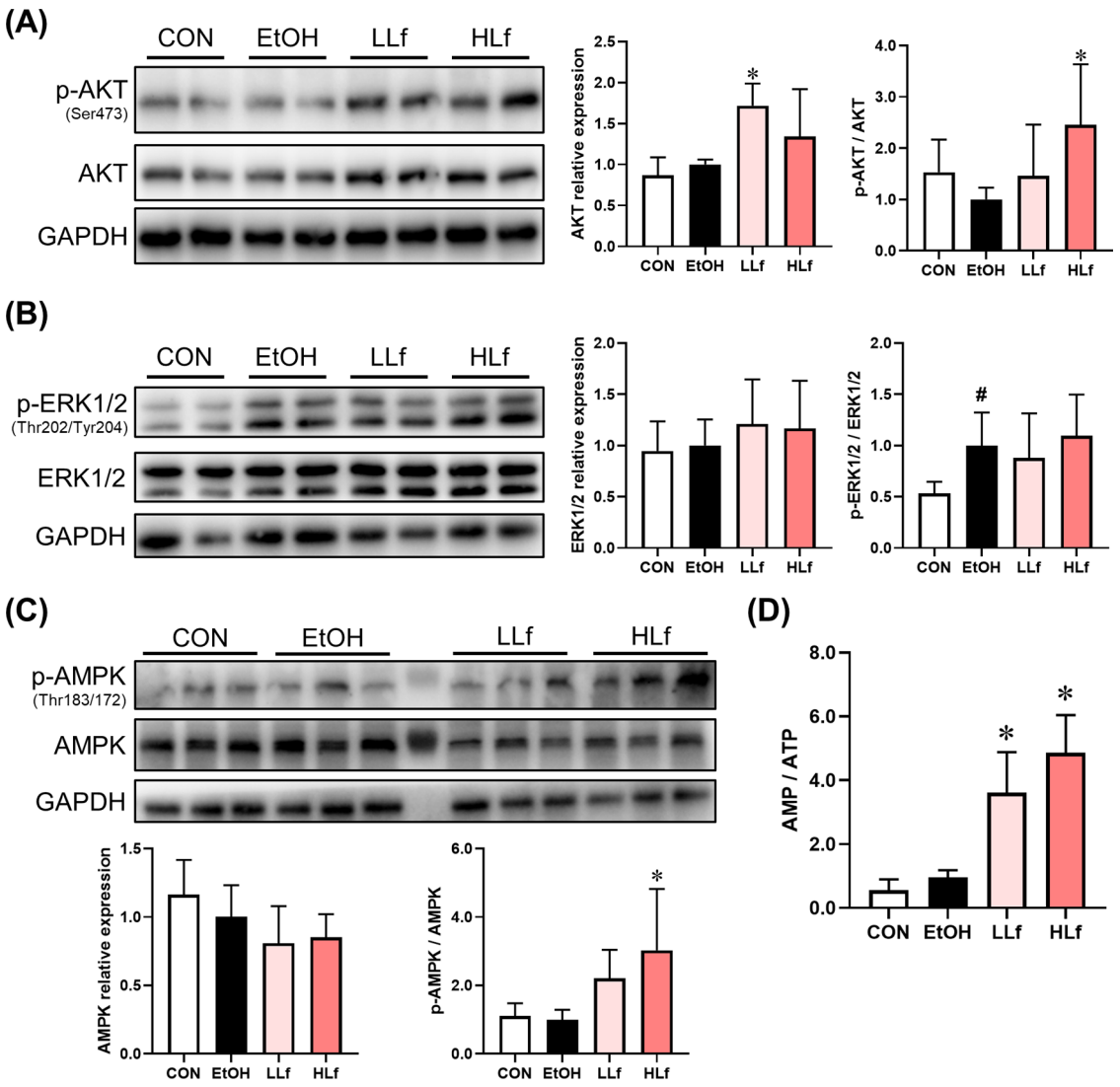

Figure 6. Effects of Lf on redox-sensitive signaling pathways. (A) Effects of Lf on AKT total protein and phosphorylation levels. (B) Effects of Lf on ERK1/2 total protein and phosphorylation levels. (C) Effects of Lf on AMPK total protein and phosphorylation levels. (D) Effects of Lf on the hepatic AMP/ATP ratio. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N \geq 6$, for each group. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

We further investigate the effects of Lf on the antioxidant system. By Lf pretreatment, not only the hepatic Nrf2 protein level was increased but also its inhibitor Keap1 expression was downregulated.[49] Meanwhile, hepatic CAT activity was significantly higher in the HLf group than in the EtOH group. Many studies have reported a suppression effect of alcohol intake on SOD activity,[50−52] which was further confirmed in this study. In addition, GPx activity upregulation might be a compensatory response for ethanol-induced SOD suppression. However, the determined activities of antioxidant enzymes did not correspond fully to their catalytic substrates. Nonetheless, the current results still supported that the antioxidant system, from a holistic perspective, could be activated by HLf pretreatment.

PI3K, as an upstream protein of AKT, also plays a role in oxidative stress response.[53,54] We observed a significant PI3K upregulation in the HLf group when compared with the EtOH group. Alcohol consumption can disrupt mitochondrial fatty acid β-oxidation, which is an important reason for ALI.[55−57] In this study, we also found that alcohol intervention caused a significant reduction in fatty acid β-oxidation key enzyme CPT1A protein levels and it was restored by Lf in a dose-dependent manner. The results further supported the protective role of HLf in acute ALI for female mice.

The damaged proteins can be eliminated via autophagy or chaperones or proteasomes.[58−61] We preliminarily explored that which elimination mechanism might play a more crucial role. Given that HLf pretreatment had a more pronounced preventive effect, we focused on the effects of HLf on these pathways. According to hepatic LC3, p62, HSP60, HSP90, PSMD13, and PSME1 protein expression levels, in the degraded protein removal process, autophagy might occupy a relatively more important position, which was supported by the increased LC3-II expression and the reduced p62 level. Some studies emphasized the role of HSP in liver diseases.[62] However, our present study found that alcohol did not affect hepatic HSP expression levels, and HLf pretreatment only had a weak effect on HSP protein expression, which suggested that acute ethanol intake-induced liver injury was not dependent on HSP, and HSP merely played a dispensable role in Lf-mediated hepatopro-

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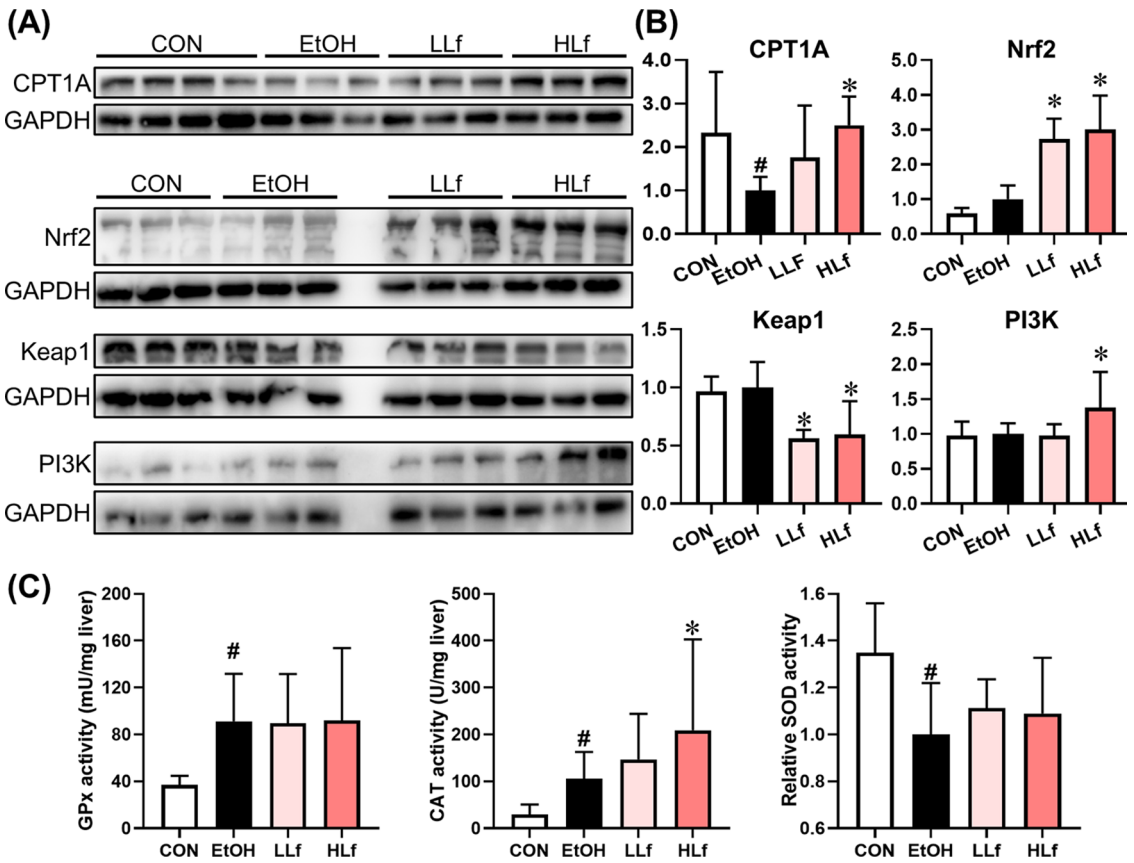

Figure 7. Effects of Lf on CPT1A, Nrf2, Keap1, and PI3K protein levels and antioxidant enzyme activities in the liver. (A) Representative western blot images. (B) Relative protein expression levels of CPT1A, Nrf2, Keap1, and PI3K. (C) Effects of Lf on hepatic antioxidant enzyme activities. CON, control group; EtOH, ethanol administration group; LLf, low-dose lactoferrin group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N \geq 4$, for each group. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

tective effects. Alcohol administration suppressed proteasome system activation, whereas HLf did little to restore proteasome activity. It is worthwhile to note that the upstream protein expression levels are not absolutely equal to the ultimate biological effects. Therefore, the conclusion that HLf promotes the degraded protein elimination mainly via autophagy is relatively preliminary and it still needs further verification.

Redox stress includes not only oxidative stress but also reductive stress.[63,64] On the one hand, oxidative stress but not reductive stress plays a crucial role in acute ALI,[16] namely, redox stress is basically completely manifested as oxidative stress in the acute ALI pathogenesis. On the other hand, Lf, as a natural food component, is unlikely to induce reductive stress, such an extreme condition. Therefore, we did not conduct the reductive stress-relevant experiments. Our previous study reported that gut microbiota modulation by Lf also plays a role in ALI in male mice, although it is weak.[12] In the present study, we did not investigate gut microbiota based on the following three considerations. First, the effects of Lf on gut microbiota are usually relatively weak, at least in the studies conducted by our research group.[12] Second, the effects of gut microbiota on health should be smooth and continuous, so the impact of gut microbiota on the acute diseases may be limited. Third, alcohol intervention was performed at the end of the experiment, and no adverse factor for "healthy" gut microbiota was introduced during the whole pretreatment period. Therefore, we think gut microbiota plays little role in Lf-mediated acute ALI amelioration in female mice.

We demonstrated theoretically that even high-dose Lf intake unlikely leads to an increase of iron overintake or iron overload in the previous study; however, experimental evidence was still absent.[12] In the present study, we assessed hepatic iron accumulation by Perls staining, and the results showed that the HLf supplement did not entail the iron overload risk.

Our study indicated that RRC elevation may play an important role in acute ALI prevention in female mice. However, there were still some limitations. First, the reasons for sexual dimorphism of the Lf-optimized dose were not illuminated. Second, the causality between RRC and acute ALI was not fully elaborated, which might need further verifying by in vivo or in vitro experiments. Third, the effective dose (equal to a woman drinking 5000 mL of milk every day) was too high to achieve through regular diet. Although dietary supplement might be a feasible strategy, such a high dose, to a certain extent, restricted practical application of Lf. In addition, potential risk of the long-term high dose of Lf intake also must be further assessed.[65,66] Fourth, our study focused on the preventive effects of Lf on liver injury induced by simple acute alcohol administration; however, alcohol drinking usually coexists with the NAFLD risk factors in the real world.[11] Thus, it is necessary to assess the roles of Lf in ALI prevention for the NAFLD model in the future. Finally, ALI is multifactorial and complex pathological process, and the mechanisms underlying ALI are far from being completely clarified. Our study only explained the acute ALI preventive effect of Lf from the perspective of RRC.

https://doi.org/10.1021/acs.jafc.1c06813
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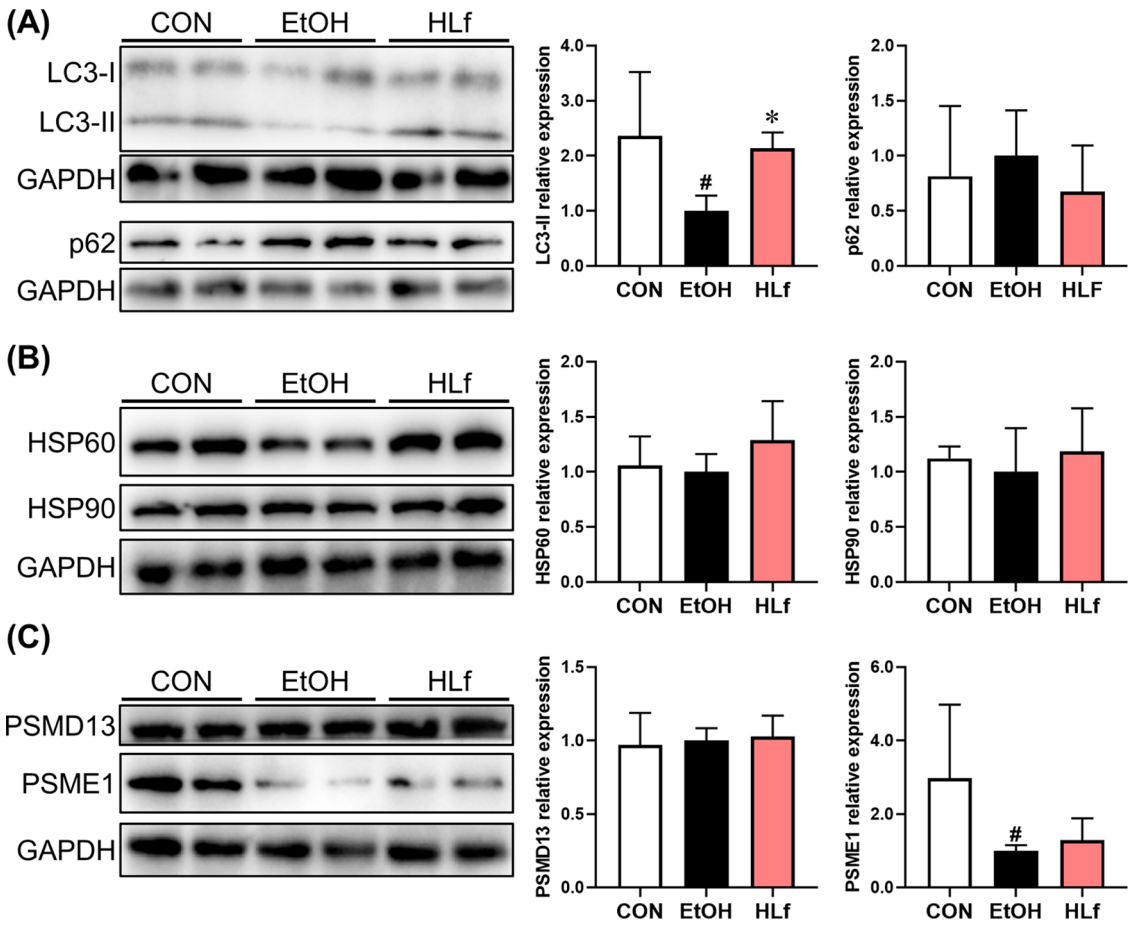

Figure 8. Effects of Lf on the damaged protein elimination system. (A) Effects of Lf on autophagy key proteins LC3 and p62. (B) Effects of Lf on chaperons HSP60 and HSP90. (C) Effects of Lf on proteasome biomarkers PSMD13 and PSME1. CON, control group; EtOH, ethanol administration group; and HLf, high-dose lactoferrin group. Data are presented as "mean ± SD". $N \geq 4$, for each group. # EtOH versus CON, $P < 0.05$; * LLf or HLf versus EtOH, $P < 0.05$.

More systematic mechanism studies should be performed in the future.

In conclusion, our study demonstrated the preventive effects of HLf on acute ALI in female mice, which was associated with the RRC improvements. The potential mechanisms are displayed in Figure 9. HLf pretreatment could increase the sensitivity of the redox-regulation signal pathway to ROS, activate the antioxidant system, and remove damaged proteins by autophagy, which led to RRC elevation and ultimately contributed to acute ALI prevention. Thus, our present study offered experimental evidence to support the benefits of Lf to acute ALI in female. However, we still should hold a circumspect attitude to Lf supplement until its safety and effectiveness are confirmed by clinical trials.

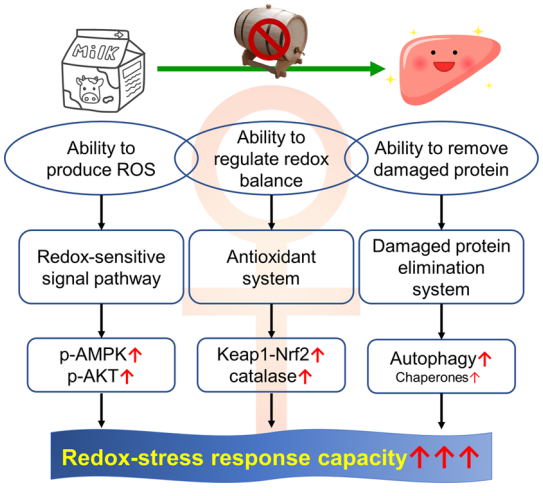

Figure 9. Diagrammatic sketch for the potential mechanisms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c06813.

The targeted metabolomics methods (Supplementary File 1) (PDF)

The KEGG analysis results (Supplementary File 2) (XLSX)

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https://doi.org/10.1021/acs.jafc.1c06813
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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank IMGBIN (https://imgbin.com/) for the supplies of the drawing materials of the graphic abstract. This work was supported by grants from the National Natural Science Foundation of China [No. 82173502, 81973024, and 82073482]. This study was also supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

■ ABBREVIATIONS USED

AALD, alcohol-associated liver disease; Lf, lactoferrin; NAFLD, nonalcoholic fatty liver disease; ALI, alcoholic liver injury; ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; RRC, redox-stress response capacity; CON, control; EtOH, ethanol; LLf, low-dose lactoferrin; HLf, high-dose lactoferrin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; HE, hematoxylin and eosin; ESR, electron spin resonance; KEGG, Kyoto Encyclopedia of Genes and Genomes; SD, standard deviation; FMN, flavin mononucleotide; GDP, guanosine diphosphate; AMPK, AMP-activated protein kinase; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; CPT1A, carnitine palmitoyltransferase 1A; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; PI3K, phosphatidylinositol 3-kinase; LC3, microtubule-associated protein light chain 3; p62, sequestosome 1; HSP, heat shock protein; PSMD13, proteasome 26S subunit non-ATPase 13; PSME1, proteasome activator subunit 1.

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