

Preventing Gut Leakiness and Endotoxemia Contributes to the Protective Effect of Zinc on Alcohol-Induced Steatohepatitis in Rats^{1–3}

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Abstract

Background: Zinc deficiency has been well documented in alcoholic liver disease.

Objective: This study was undertaken to determine whether dietary zinc supplementation provides beneficial effects in treating alcohol-induced gut leakiness and endotoxemia.

Methods: Male Sprague Dawley rats were divided into 3 groups and pair-fed (PF) Lieber-DeCarli liquid diet for 8 wk: 1) control (PF); 2) alcohol-fed (AF; 5.00–5.42% wt:vol ethanol); and 3) AF with zinc supplementation (AF/Zn) at 220 ppm zinc sulfate heptahydrate. The PF and AF/Zn groups were pair-fed with the AF group. Hepatic inflammation and endotoxin signaling were determined by immunofluorescence and quantitative polymerase chain reaction (qPCR). Alterations in intestinal tight junctions and aldehyde dehydrogenases were assessed by qPCR and Western blot analysis.

Results: The AF rats had greater macrophage activation and cytokine production ($P < 0.05$) in the liver compared with the PF rats, whereas the AF/Zn rats showed no significant differences ($P > 0.05$). Plasma endotoxin concentrations of the AF rats were 136% greater than those of the PF rats, whereas the AF/Zn rats did not differ from the PF rats. Ileal permeability was 255% greater in the AF rats and 19% greater in the AF/Zn rats than in the PF rats. The AF group had reduced intestinal claudin-1, occludin, and zona occludens-1 (ZO-1) expression, and the AF/Zn group had upregulated claudin-1 and ZO-1 expression ($P < 0.05$) compared with the PF group. The intestinal epithelial expression and activity of aldehyde dehydrogenases were elevated ($P < 0.05$) in the AF/Zn rats compared with those of the AF rats. Furthermore, the ileal expression and function of hepatocyte nuclear factor 4 α , which was impaired in the AF group, was significantly elevated in the AF/Zn group compared with the PF group.

Conclusions: The results demonstrate that attenuating hepatic endotoxin signaling by preserving the intestinal barrier contributes to the protective effect of zinc on alcohol-induced steatohepatitis in rats. *J Nutr* 2015;145:2690–8.

Keywords: alcohol, zinc, intestinal barrier, endotoxemia, aldehyde dehydrogenase, HNF-4 α , alcoholic liver disease

Introduction

Cytokine production in the liver is a critical mediator of the initiation and progression of alcoholic liver disease (ALD)⁷ because it induces inflammation and cell death (1, 2). Many factors such as endotoxins (LPS) stimulate the production of cytokines in the liver

(3, 4). Indeed, increased blood endotoxin concentrations resulting from gut leakiness have been suggested to be a causal factor in triggering hepatic cytokine production (5). Significantly increased blood endotoxin concentrations were found in alcoholic patients with different stages of liver injury, including steatosis, hepatitis, and cirrhosis and in experimental animal models of ALD (5–7). Moreover, it has been reported that blood endotoxin concentrations strongly correlate with TNF- α concentrations in alcoholic cirrhosis (8, 9). In a clinical study of patients with alcoholic hepatitis over a 6-mo hospitalization period, the serum IL-8, TNF- α , and bilirubin concentrations correlated with the severity of liver damage (10).

Disruption of the intestinal barrier has been suggested to be a leading cause of endotoxemia in ALD (5). Clinical studies have shown that only alcoholics with leaky gut syndrome develop ALD (11). We have reported that orally administered LPS can

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³ Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://nutrition.org>.

⁷ Abbreviations used: AF, alcohol-fed; ALD, alcoholic liver disease; ALDH, aldehyde dehydrogenase; *Cinc1*, cytokine-induced neutrophil chemoattractant 1; HNF-4 α , hepatocyte nuclear factor 4 α ; *Mcp1*, monocyte chemoattractant protein 1; PF, pair-fed; ZO-1, zona occludens-1.

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be detected in the plasma of alcohol-intoxicated mice but not in control mice, providing direct evidence that alcohol exposure increases gut permeability to endotoxins (12). Under normal physiological conditions, only trace amounts of endotoxins can penetrate the blood stream from the intestinal lumen because of the intestinal epithelial barrier (5). Tight junctions are the major components responsible for epithelial barrier formation by sealing the intercellular spaces between adjacent epithelial cells (13). Animal studies have shown that the prevention of gut leakiness can suppress alcohol exposure-induced endotoxemia and liver damage (12, 14). Therefore, preventing the leaky gut is likely an attractive therapeutic strategy for treating ALD.

Zinc is the second most abundant trace element in the body after iron (15). It plays a key role in maintaining all major physiological functions in the body, such as metabolism, signal transduction, cell growth, and differentiation (16). Zinc deficiency has been well documented both in alcoholic patients and experimental models of ALD (17–19). The severity of hepatic reduction of zinc concentration tends to increase as ALD progresses from alcoholic steatosis to cirrhosis (20). In particular, our previous study found that alcohol-induced zinc deficiency critically modulated intestinal barrier dysfunction and the development of endotoxemia and liver injury in mice (19). We also reported that the inactivation of intestinal hepatocyte nuclear factor 4 α (HNF-4 α) as a result of zinc deficiency mediates alcohol-induced downregulation of intestinal tight junction proteins (21). Moreover, we found that marginal dietary zinc deficiency exaggerated alcohol-induced gut hyperpermeability, endotoxemia, and alcoholic liver injury (6).

Dietary zinc supplementation has been reported to prevent and reverse alcohol-induced liver injury. Our previous study reported that zinc supplementation reversed alcoholic steatosis in mice by restoring the function of zinc finger transcription factors HNF-4 α and PPAR- α in the liver (22). However, the mechanisms regarding the effects of zinc supplementation on the intestine during the progression of ALD have not been fully elucidated. Therefore, we sought 1) to determine whether dietary supplementation with zinc provides protection against alcohol-induced gut leakiness and endotoxemia and 2) to define the molecular mechanisms of how zinc modulates intestinal barrier function and acetaldehyde detoxification.

Methods

Animals and treatments. All treatments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus. Male Sprague Dawley rats (Charles River) aged 3 mo were pair-fed (PF) a modified Lieber-DeCarli liquid diet (23) containing alcohol (alcohol-fed, AF; $n = 8$) or isocaloric maltose dextrin as a control (PF, $n = 6$) for 8 wk. Both the PF and AF diets (Dyets) contained 14 mg of zinc carbonate per liter (7.3 mg of elemental zinc per liter). In the AF/Zn group ($n = 8$), 200 mg/L zinc sulfate heptahydrate (45 mg of elemental zinc per liter) was added to the alcohol diet. The wt:vol ethanol content (Sigma-Aldrich) was gradually increased from 5% for the first 2 wk to 5.42% for the last 2 wk, increasing by 0.14% every 2 wk (Supplemental Table 1). Because rats in the AF group consumed the least amount of food, they consumed food ad libitum, and the PF and the AF/Zn rats were pair-fed the same amount of diet that the AF rats consumed during the previous day. At the end of the experiment, rats were anesthetized with isoflurane, and plasma, liver, intestinal mucosa, and intestinal contents were collected for assays.

Histochemical staining of hepatic lipids. To detect lipid accumulation in the liver, cryostat liver sections were stained following the oil red O staining procedure as described previously (6).

Immunofluorescence procedure. To detect macrophages or tight junction proteins, cryostat sections of the liver were incubated with anti-CD68 or anti-CD163 (BD Biosciences), and cryostat sections of the ileum and colon were incubated with anti-occludin or anti-zona occludens-1 (ZO-1; Millipore) antibodies overnight at 4°C, respectively, followed by incubation with Alexa Fluor 594-conjugated donkey anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min at room temperature.

Immunoperoxidase procedure. The localization of HNF-4 α in the ileum was determined by immunohistochemical staining as described previously (21).

Measurement of zinc concentrations in the liver and plasma by atomic absorption spectrophotometry. Zinc concentrations in the liver and plasma were measured by atomic absorption spectrophotometry as described previously (17). The measured zinc concentrations were calculated as $\mu\text{g/g}$ dry liver weight and $\mu\text{g/dL}$ for the liver and plasma, respectively.

Endotoxin assay. The endotoxin concentrations in the liver and intestinal contents were assessed by a chromogenic kinetic limulus amoebocyte lysate assay kit (Lonza) following the manufacturer's instructions (19). The concentration of endotoxin was expressed in endotoxin units/mL for plasma and endotoxin units/mg weight for intestinal contents.

Determination of ileal permeability. Ileal permeability was assessed by analyzing the penetration rate of FD4 fluorescein isothiocyanate-dextran (Sigma-Aldrich) from freshly isolated ileum sacs as described previously (19).

Ethanol and acetaldehyde concentrations within the intestinal lumen. The concentrations of ethanol and acetaldehyde within the intestinal lumen were measured by headspace gas chromatography-mass spectrometry as described previously (24, 25).

Assessment of aldehyde dehydrogenase activity. Aldehyde dehydrogenase (ALDH) activity was measured by a commercial kit (BioVision) following the manufacturer's instructions. Briefly, acetaldehyde was oxidized by the ALDHs in the homogenates to nicotinamide adenine dinucleotide, which further reduced a colorless probe to a colored product with strong absorbance at 450 nm.

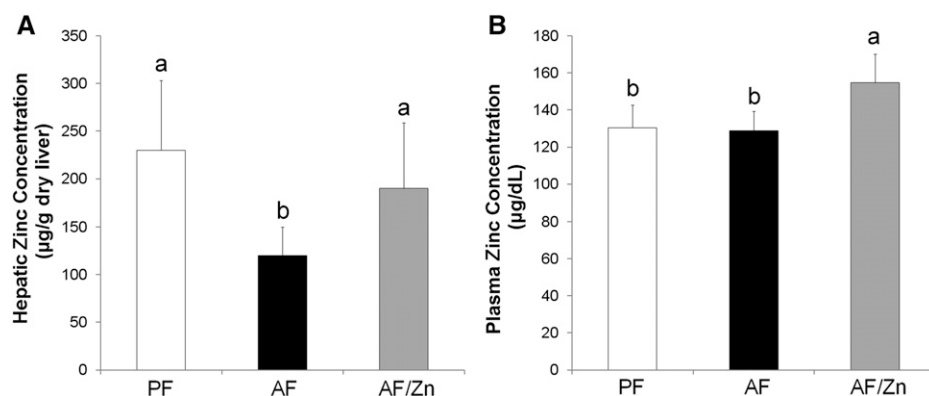
Assessment of HNF-4 α activity. The ileal HNF-4 α activity was assessed by measuring the DNA binding ability with an ELISA kit (Active Motif) (21).

qPCR analysis. Total RNA was isolated from the liver or ileum mucosa and reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems). The gene expression of related mRNA was measured in triplicate by the comparative cycle threshold method using a 7500 real-time PCR system (Applied Biosystems). The primer sequences (Integrated DNA Technologies) are shown in Supplemental Table 2. The data were normalized to 18S rRNA mRNA levels and presented as fold changes, setting the value of the PF rats as 1.

Western blot analysis. The preparation of ileal protein lysates and the Western blot analysis were conducted as described previously (7). Antibodies used for the Western blot included anti-ALDH2 (Abcam); anti-ALDH1B1 (Santa Cruz Biotechnology); anti- β -actin (Sigma-Aldrich); and anti-ALDH1A1, anti-ALDH9A1, and anti-HNF-4 α (Novus Biologicals). The bound complexes were detected with enhanced chemiluminescence and quantified by densitometry analysis.

Statistical analysis. All data are expressed as mean \pm SD. For 3-group comparisons, the data were analyzed by 1-way ANOVA followed by Newman-Keuls post hoc test. For 2-group comparisons, Student's t test was used. All data were tested for homogeneity of variance. For unequal variances, data were evaluated by the Kruskal-Wallis test. Differences between groups were considered significant at $P < 0.05$.

FIGURE 1 Hepatic (A) and plasma (B) zinc concentrations in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; PF, pair-fed.



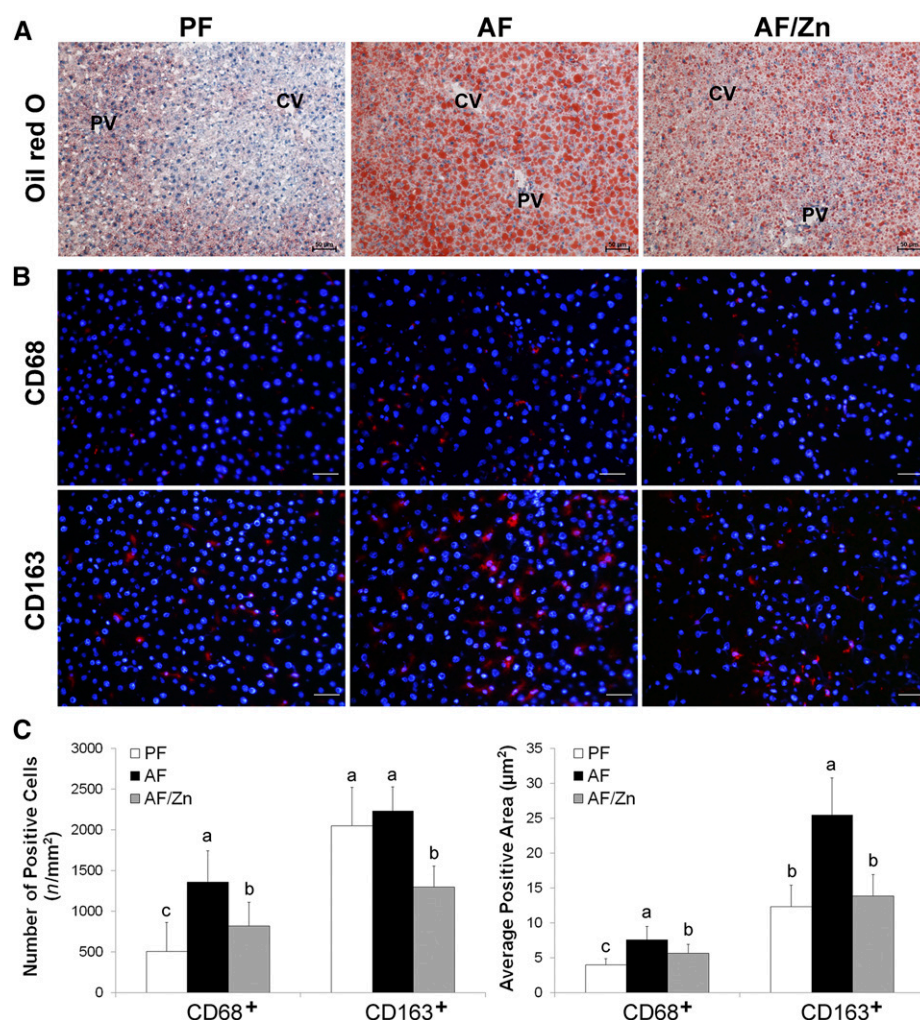
Results

Body weight, liver weight, and liver histopathological changes. There was no significant difference in the initial mean body weights of rats among the groups (319.8 ± 16.5 g, $P = 0.87$). The AF rats consumed food ad libitum, whereas the PF and AF/Zn rats were pair-fed the same amount that the AF rats consumed the previous day (mean daily food intake was 67.5 ± 2.1 g, $P = 0.95$). After 8 wk of feeding, the average body weights of rats were comparable among the 3 groups (397.7 ± 26.1 g, $P = 0.98$). Liver weights, however, were significantly increased in the AF groups compared with that in the PF group (10.6 ± 0.8 g

for the PF group, 12.9 ± 1.0 g for the AF group, and 12.9 ± 0.6 g for the AF/Zn group; $P < 0.001$). Hepatic zinc concentration was significantly reduced in the AF rats compared with the PF rats and normalized by zinc supplementation (Figure 1A). Compared with the PF and AF rats, the AF/Zn rats had elevated plasma zinc concentrations ($P < 0.05$) (Figure 1B).

The AF group showed excessive lipid accumulation (Figure 2A) and macrophage activation (Figure 2B) in the liver compared with the PF group. Quantification analysis of positively stained macrophage subtypes showed that the number of CD68⁺ macrophages, but not CD163⁺ macrophages, increased in the AF rats

FIGURE 2 Hepatic lipid accumulation and macrophage activation in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. (A) Hepatic lipid accumulation stained by oil red O. The nuclei were counterstained by hematoxylin. Scale bar: 50 μ m. (B) Immunofluorescent staining of hepatic macrophages. Scale bar: 20 μ m. Red: CD68⁺ or CD163⁺ macrophages; blue: 4',6-diamidino-2-phenylindole counterstaining of the nuclei. (C) Quantitative analysis of CD68⁺ or CD163⁺ macrophages in the liver ($n = 25-35$). Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; CV, central vein; PF, pair-fed; PV, portal vein.



compared with the PF rats, and the cell sizes of both subtypes were enlarged (Figure 2C). Zinc supplementation reduced hepatic lipid accumulation in the AF/Zn rats compared with the AF rats (Figure 2A, B). Moreover, the number of CD68⁺ cells in the AF/Zn rats was greater than that in the PF rats but less than that in the AF rats, whereas the number of CD163⁺ cells was less than that in both the PF and AF rats ($P < 0.05$) (Figure 2C). The mean area with CD68⁺-positive staining in the AF/Zn rats was greater than that in the PF rats but less than that in the AF rats. Furthermore, the mean area of CD163⁺-positive staining was less than that in the AF rats and comparable to that in the PF rats ($P < 0.05$) (Figure 2C).

Endotoxemia, hepatic LPS signaling, and intestinal barrier function. Plasma endotoxin concentrations and ileal permeability were measured to determine whether the prevention of alcoholic liver injury by zinc occurs through the inhibition of endotoxemia. As shown in Figure 3A, alcohol exposure elevated the plasma endotoxin concentration by >100% compared with the PF rats, whereas zinc supplementation prevented this increase. Zinc supplementation also benefited the alcohol-impaired intestinal barrier function, as indicated by significantly lowered ileal permeability in the AF/Zn rats compared with that of the AF rats, which exhibited a 255% elevation compared with the PF rats ($P < 0.001$) (Figure 3B). In accordance with these findings, zinc attenuated the alcohol-induced upregulation of hepatic LPS signaling molecules, including *Cd14* and LPS-binding protein (*Lbp*) (Figure 3C, D). Furthermore, compared with the PF group, hepatic cytokine-induced neutrophil chemoattractant 1 (*Cinc1*) and monocyte chemoattractant protein 1 (*Mcp1*) were upregulated in the AF group and normalized in the AF/Zn group (Figure 3E, F).

Intestinal tight junctions. The intestinal epithelial tight junctions were examined to explore how zinc modulates the intestinal barrier. As shown in Figure 4A, chronic alcohol exposure significantly decreased the mRNA levels of several tight junction proteins, including *claudin1*, *occludin*, and *Zo1*, in the ileum and colon. Zinc supplementation significantly upregulated the expression of claudin-1 in both the ileum and colon and upregulated ileal *Zo1* expression. The distributions of occludin and ZO-1 were estimated by immunofluorescent staining. In contrast to the PF group, which showed clear and uniform positive staining of the tight junction proteins at the apical part of the intestinal epithelium, the AF group showed reduced staining of both occludin and ZO-1 in the ileum and colon (Figure 4B). These pathophysiological changes were improved in the AF/Zn rats (Figure 4B).

Intestinal aldehyde hydrogenases. The intestinal response to ethanol in the presence and absence of zinc supplementation was determined because the intestine is the first site of injury after exposure to ethanol and its metabolites. Alcohol consumption robustly increased the intestinal luminal ethanol (Figure 5A) and acetaldehyde concentrations (Figure 5B) in both the ileum and colon. However, zinc supplementation had no effect on the intestinal luminal ethanol or acetaldehyde concentrations. The expression levels of intestinal ALDHs were measured to explore the response of the intestine to alcohol intoxication. Among the 14 ALDHs tested, the mRNA levels of *Aldh1a3*, *Aldh1b1*, *Aldh3a2*, *Aldh5a1*, *Aldh7a1*, and *Aldh8a1* were significantly decreased, and the mRNA levels of *Aldh2* and *Aldh4a1* were increased in the ileum after alcohol exposure. The mRNA levels

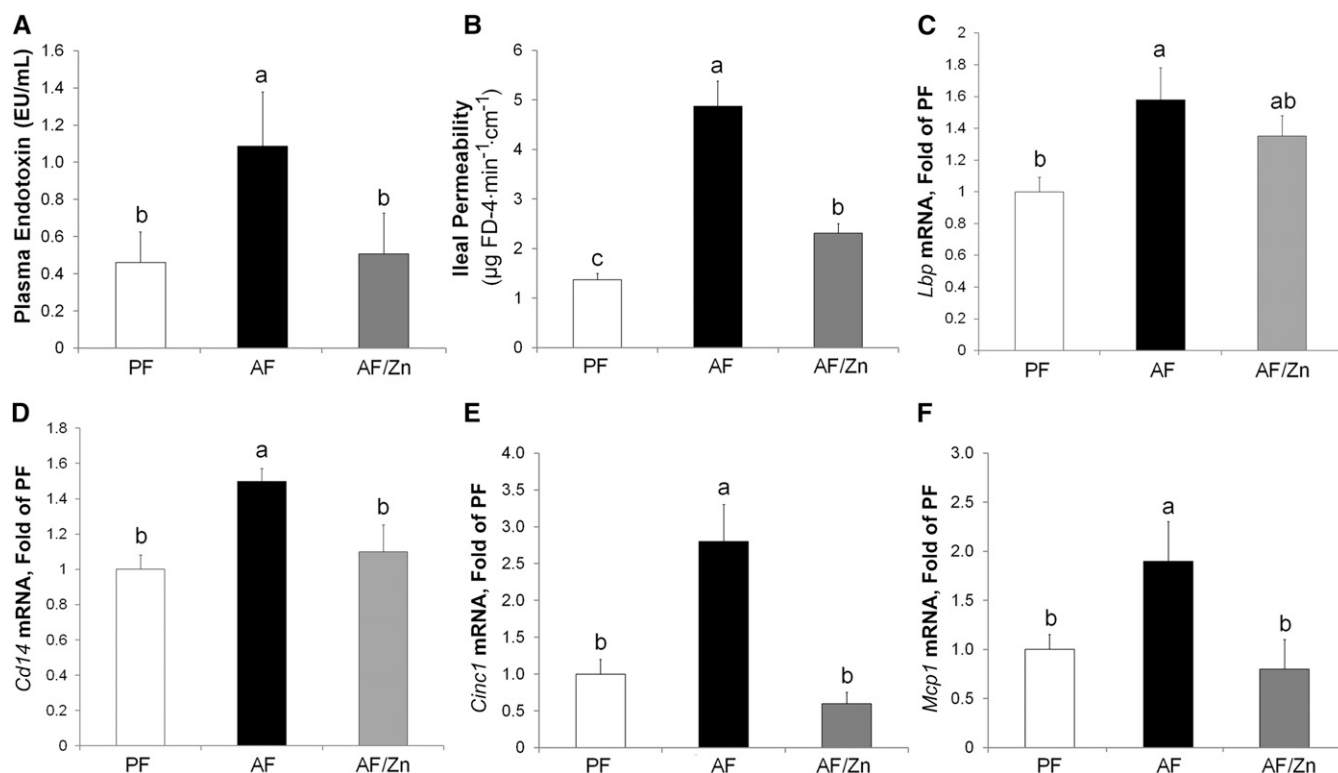


FIGURE 3 Endotoxemia and hepatic LPS signaling in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. (A) Plasma endotoxin concentrations. (B) Ileal permeability as indicated by the leakage of intraluminal fluorescein isothiocyanate-dextran. (C, D) qPCR assay of hepatic mRNA levels of the LPS signaling molecules *Lbp* (C) and *Cd14* (D). (E, F) qPCR assay of hepatic mRNA levels of the chemokines *Cinc1* (E) and *Mcp1* (F). Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; *Cinc1*, cytokine-induced neutrophil chemoattractant 1; EU, endotoxin units; FD, fluorescein isothiocyanate-dextran; *Lbp*, LPS-binding protein; PF, pair-fed; *Mcp1*, monocyte chemoattractant protein 1.

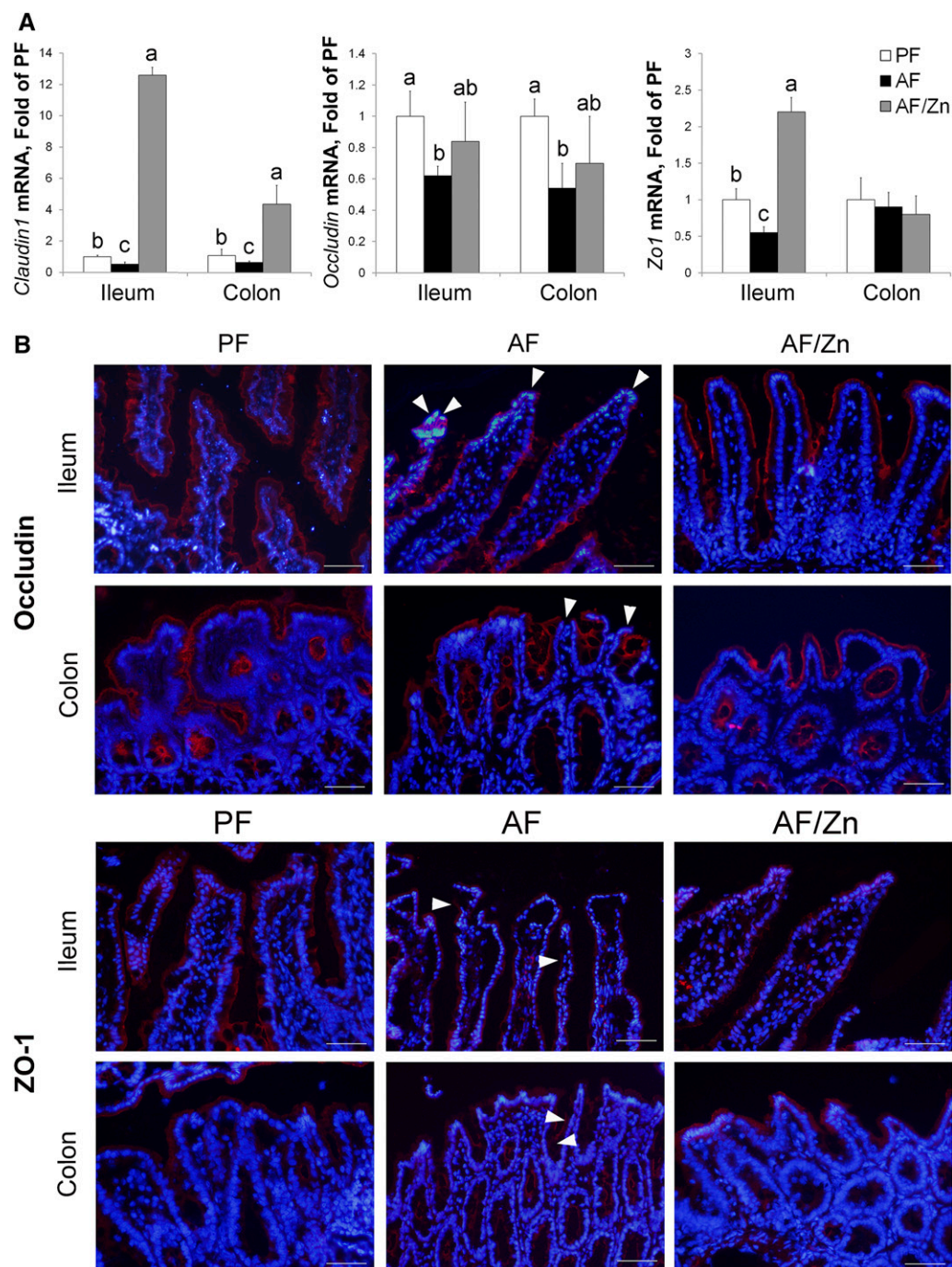


FIGURE 4 Intestinal tight junction proteins in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. (A) qPCR assay of the mRNA levels of the tight junction proteins *claudin1*, *occludin*, and *Zo1* in the ileum and colon. Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. (B) Immunofluorescent staining of occludin and ZO-1 in the ileum and colon. Scale bar: 20 μ m. Red: occludin or ZO-1; blue: 4',6-diamidino-2-phenylindole counterstaining of the nuclei; white triangle: disassembled tight junction proteins. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; PF, pair-fed; ZO-1, zona occludens-1.

of *Aldh1a1* and *Aldh1b1* were decreased, and the mRNA levels of *Aldh1a3* were increased in the colon (Figure 6A). The effect of zinc on the expression of *Aldh2*, *Aldh1a1*, and *Aldh1b1* was further assessed. As shown in Figure 6B, zinc upregulated the expression of all the ALDHs in the colon, whereas *Aldh1a1* mRNA levels were elevated in the ileum. In agreement with these findings, the ALDH activities were elevated both in the ileum and colon of the AF/Zn rats compared with the AF rats (Figure 6C). Western blot analysis of the ileal ALDH proteins showed that ALDH1A1 was reduced, whereas ALDH1B1 and ALDH9A1 were increased after alcohol

feeding. Zinc supplementation significantly increased the ileal ALDH1A1 and ALDH1B1 to the highest levels among the 3 groups but prevented alcohol-induced increases in ALDH9A1. Moreover, the ileal ALDH2 protein concentrations were not affected by either alcohol or zinc treatment (Figure 6D, E).

Intestinal HNF-4 α . The expression of transcription factor HNF-4 α , which is a zinc-finger protein, in the ileum was measured to investigate the molecular mechanisms of zinc-mediated modulation of intestinal tight junction proteins.

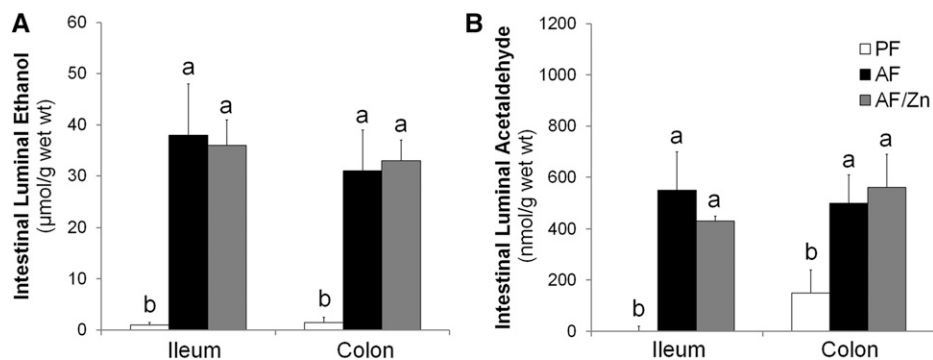


FIGURE 5 Intestinal luminal concentrations of ethanol (A) and acetaldehyde (B) in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; PF, pair-fed.

Immunohistochemical staining of ileal HNF-4 α showed that chronic alcohol exposure reduced the distribution of HNF-4 α in the epithelial nuclei of ileal villi and crypts, particularly in the latter (Figure 7A). The apical epithelium of the villi was also severely damaged by alcohol exposure, and the alcohol-mediated

histopathological changes were improved by zinc supplementation. The protein concentrations of ileal HNF-4 α were significantly decreased in the AF group compared with the PF group ($P < 0.05$) and significantly increased in the AF/Zn group compared with the AF group ($P < 0.01$) (Figure 7B). In addition,

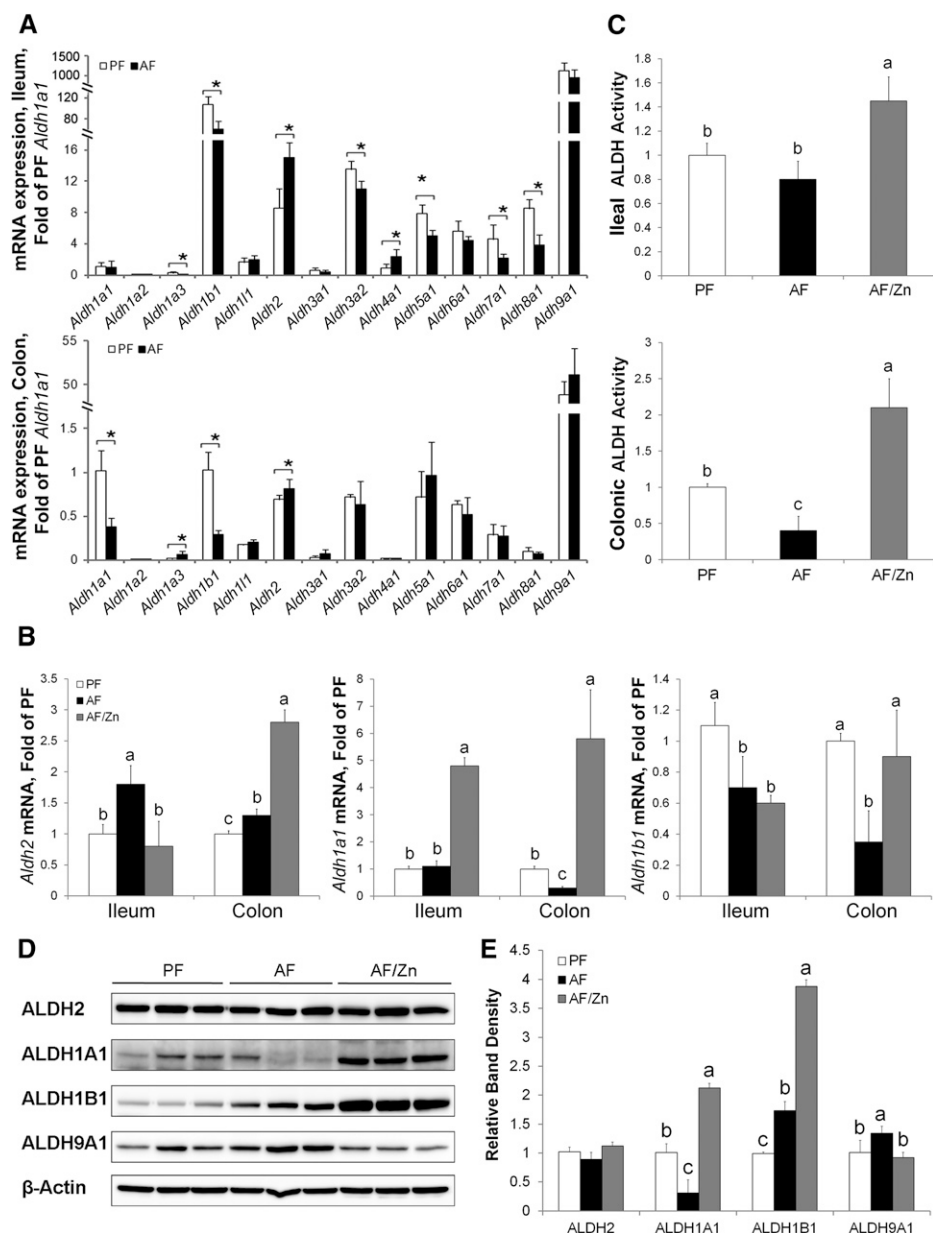


FIGURE 6 Intestinal ALDHs in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. (A) qPCR assay of the *Aldh* mRNA levels in the ileum and colon. *Different from PF, $P < 0.05$ (Student's t test). (B) qPCR assay of the mRNA levels of *Aldh2*, *Aldh1a1*, and *Aldh1b1* in the ileum and colon. (C) Ileal and colonic ALDH activity. (D) Western blots of ileal ALDH2, ALDH1A1, ALDH1B1, and ALDH9A1. (E) Relative density of the Western blot bands. Results are mean \pm SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$, for panels B-E. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; ALDH, aldehyde dehydrogenase; PF, pair-fed.

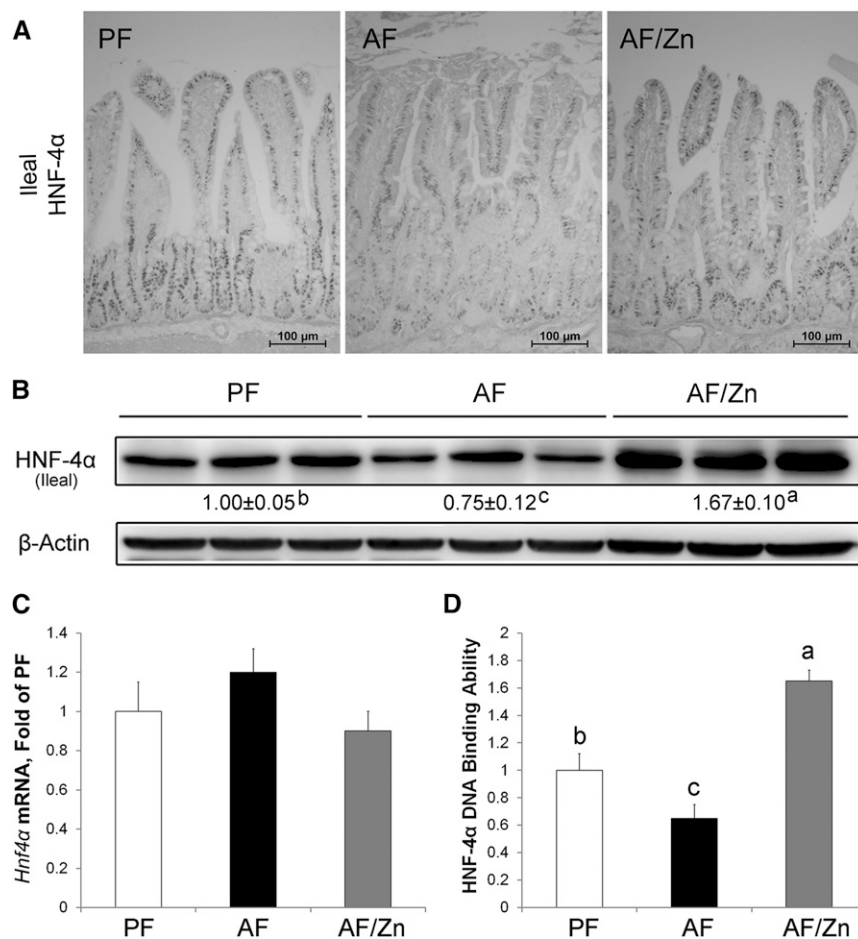


FIGURE 7 Ileal HNF-4α in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. (A) Immunohistochemical staining of HNF-4α in the ileum. Scale bar: 100 μm. (B) Western blot of ileal HNF-4α. (C) qPCR assay of the mRNA levels of ileal *Hnf4a*. (D) Ileal HNF-4α activity. Results are mean ± SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; HNF-4α, hepatocyte necrosis factor 4α; PF, pair-fed.

the increased protein concentrations of HNF-4α did not result from regulation at the transcription level because no differences in the mRNA levels of *Hnf4a* were found among the 3 groups (Figure 7C). As indicated by decreased DNA binding ability compared with the PF controls, the activity of HNF-4α was impaired by alcohol exposure, whereas zinc supplementation significantly increased ileal HNF-4α function (Figure 7D).

Intestinal luminal endotoxin concentrations. The effects of ethanol and zinc on intestinal luminal endotoxin concentrations were also determined. As shown in Figure 8, alcohol exposure did not alter the overall endotoxin concentrations within the intestine. Strikingly, dietary supplementation with zinc significantly reduced the luminal endotoxin concentrations both in the ileum and colon compared with the AF group ($P < 0.05$).

Discussion

Hypo-zincemia (i.e., low serum and hepatic zinc concentrations) has been well documented in ALD (15, 17–19). Importantly, the severity of hepatic zinc reduction tends to increase as ALD progresses from alcoholic steatosis to cirrhosis (20), which strongly indicates a vital role of zinc homeostasis in the pathogenesis of the disease. Multiple studies have demonstrated that dietary zinc supplementation can effectively compensate for acquired zinc deficiency in ALD (26, 27). Our previous study reported that reduced plasma zinc concentrations returned to normal concentrations in mice that consumed alcohol during 16 wk after zinc sulfate was administered during the last 4 wk of the

study (22). In this study we administered zinc sulfate, 1 of the most widely used forms of dietary zinc supplementation, to alcohol-fed rats and found that dietary supplementation of zinc sulfate for 8 wk effectively normalized the reduced hepatic zinc concentrations caused by alcohol exposure. However, alcohol consumption did not affect the plasma zinc concentrations in our

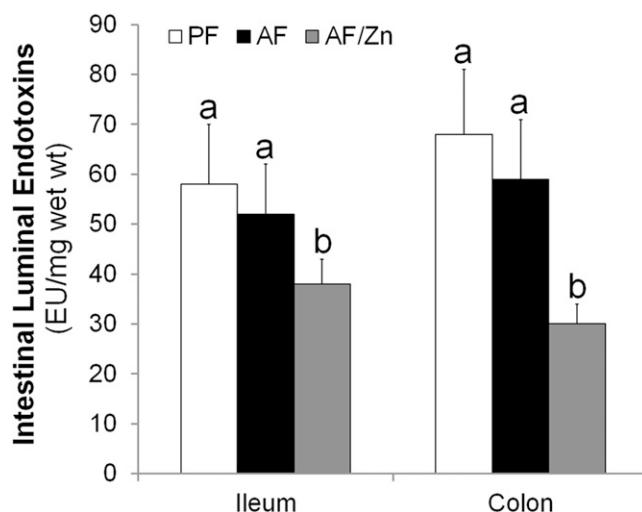


FIGURE 8 Intestinal luminal endotoxin concentrations in rats fed a control diet or an alcohol-containing diet with or without zinc supplementation for 8 wk. Results are mean ± SD ($n = 6-8$). Labeled means without a common letter differ, $P < 0.05$. AF, alcohol-fed; AF/Zn, alcohol-fed plus zinc; PF, pair-fed.

model. Hartoma et al. (28) reported that serum zinc concentrations were elevated in alcoholic subjects with normal or fatty livers, whereas reduced serum zinc concentrations were found in patients with alcoholic hepatitis or cirrhosis. These data suggest that serum zinc concentrations might be elevated at early stages of ALD as a result of excessive zinc release from organs but eventually decreases at advanced stages of the disease. Therefore, blood zinc concentrations may not be a good indicator for assessing dietary and/or organ zinc status under pathophysiological conditions.

Mechanistic studies have shown that zinc functions as a hepatoprotective agent by regulating lipid homeostasis, antioxidant defense, cell proliferation, and cell death in the liver during the development and progression of ALD (15). Increasing evidence suggests that zinc also plays an important role in regulating the barrier function of the gastrointestinal tract (29–31). Oral zinc supplementation has been reported to tighten the leaky intestine and preserve the intestinal barrier function under a variety of disease conditions, such as Crohn disease, experimental colitis, malnutrition, and enteric pathogen challenge (32–35). Our previous study showed that zinc protected intestinal histopathological changes, abrogated endotoxemia, and prevented liver damage against acute alcohol intoxication (12). This study demonstrated an extrahepatic action of zinc in preventing alcohol-induced gut leakiness and endotoxemia. We also found that hepatic LPS signaling, chemokine production, and inflammatory cell activation were all attenuated by zinc intervention, suggesting a protective role of zinc in the intestine in addition to the hepatic action with regard to ALD.

This study indicates that the protection of the intestinal barrier by zinc was achieved in part through the restoration of tight junction proteins at multiple levels. First, zinc supplementation induced the expression of tight junction genes at the transcription level. Second, it prevented the disassembly of tight junctions at the apical region of the intestinal epithelium. Third, zinc supplementation increased intestinal ALDH expression and activity by increasing the expression of *Aldh2*, *Aldh1a1*, and *Aldh1b1*, which are the 3 major ALDHs involved in aldehyde clearance, especially acetaldehyde. Although the intestinal luminal alcohol and acetaldehyde concentrations were not affected by zinc, the increased epithelial ALDH activity indicates a more effective response of the intestinal epithelium to counteract the harmful effects of alcohol exposure (36). Interestingly, zinc dramatically increased the protein concentrations of ileal ALDH1B1 without affecting its expression, indicating that zinc induces certain posttranslational mechanisms to modulate intestinal ALDHs. One possibility could be the antioxidant property of zinc. Zinc is a well-known antioxidant that acts by preserving the stability and activity of zinc-containing antioxidants such as glutathione, metallothioneins, and superoxide dismutase 1 (Cu/Zn SOD) (15). Therefore, zinc may retard the oxidative processes and subsequently preserve the function of ALDHs as well as the distribution of tight junction proteins.

HNF-4 α is a zinc finger transcription factor that belongs to the nuclear hormone receptor superfamily (37). The role of HNF-4 α has been extensively studied in hepatocytes and pancreatic β -cells, although its function in the intestine remains largely unknown. Our previous study demonstrated that alcohol-induced inactivation of intestinal HNF-4 α correlates with the downregulation of tight junction proteins (21). Recently, a genome-wide association study found that HNF-4 α is a susceptibility gene for ulcerative colitis, which is a disease that exhibits altered intestinal junctional complexes and inflammation (38). In this study, the increased expression of tight junction proteins was associated with elevated intestinal

HNF-4 α activity after zinc supplementation, suggesting that HNF-4 α plays a critical role in regulating the intestinal barrier as well as in the pathogenesis of alcohol-induced endotoxemia. We also found that alcohol-induced inactivation of intestinal HNF-4 α did not occur at the transcription level. The role of zinc in the regulation of HNF-4 α is to stabilize the zinc finger structure, which is required for its protein-DNA binding capacity (39). Under oxidative stress, oxidation of a zinc-binding cysteine may release zinc, leading to the elimination of the DNA binding ability and degradation of the destabilized protein (40). Zinc release might be a key mechanism that accounts for the pathological effects of alcohol in the intestine because minute changes in the availability of cellular zinc have potent effects.

We also demonstrated for the first time, to our knowledge, that zinc reduces intestinal luminal endotoxin concentrations, suggesting that zinc may reduce endotoxin-producing bacteria to further prevent endotoxemia. Although the role of zinc on immunoregulation has long been investigated and emphasized, its antimicrobial effects are still under investigation. Zinc concentrations in tissues are increased during a *Streptococcus pneumoniae* infection, and previous studies have shown that zinc actually kills bacteria (41). Zinc supplementation has also been reported to reduce diarrhea duration and inhibit the growth of bacterial pathogens isolated from diarrheal stool specimens, including *Salmonellae*, enteropathogenic *Escherichia coli*, and *Shigellae*, which are all gram-negative bacteria that contain LPS as a major component of their outer membrane (42). Therefore, the effects of zinc supplementation on intestinal microbiota homeostasis and the relevance with regard to ALD pathogenesis need to be explored in future studies.

In conclusion, dietary zinc supplementation effectively corrects alcohol-mediated zinc deficiency and ameliorates alcoholic liver inflammation in Sprague Dawley rats. The protection of zinc against alcoholic endotoxemia is achieved at multiple levels within the intestine, leading to reduced luminal endotoxin concentrations, enhanced epithelial barrier function, and accelerated acetaldehyde clearance (summarized in **Supplemental Figure 1**). These results demonstrate that the protective actions of zinc in the intestine critically contribute to its beneficial effect against alcoholic steatohepatitis. This study also provides experimental evidence that dietary zinc supplementation has therapeutic potential for treating ALD.

Acknowledgments

W Zhong and ZZ designed the research; W Zhong and QL conducted the animal feeding; W Zhong, QL, QS, W Zhang, and XS conducted the biological experiments and analyzed the data; JZ, XY, and XZ measured the zinc concentration; W Zhong wrote the manuscript; and ZZ had primary responsibility for the final content. All authors read and approved the final manuscript.

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