Prevention of alterations in intestinal permeability is involved in zinc inhibition of acute ethanol-induced liver damage in mice

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Abstract

Acute ethanol exposure causes liver injury in experimental animals and accumulating evidence suggests that a major responsible factor for the pathogenesis is endotoxemia, which results from bacterial endotoxin leakage from the small intestine due to increased intestinal permeability under alcohol challenge. The purpose of this study was to examine whether zinc pretreatment would inhibit acute ethanol-induced liver injury through prevention of intestinal permeability changes. Male 129 Sv\textsuperscript{PCJ} mice were treated with three intragastric doses of ZnSO\textsubscript{4} at 5 mg zinc ion/kg each dosing prior to acute ethanol challenge with a single oral dose of 6 g/kg ethanol. The zinc treatment did not alter the elevation of serum concentrations of alcohol. The acute ethanol exposure caused an elevation in serum alanine aminotransferase (ALT) levels as well as fatty liver and hepatic degenerative necrotic foci as determined by biochemical assay and histochemical analysis, respectively. A significant increase in liver tumor necrosis factor-alpha (TNF-\textalpha) levels was detected by ELISA. These pathological effects correlated well with increases in serum endotoxin levels. Importantly, acute ethanol treatment caused significant damage to the small intestine as determined by morphological analysis of intestinal sections and permeability assay. These alcohol-induced hepatic pathological changes and TNF-\textalpha elevation were significantly inhibited in the zinc-pretreated animals. The inhibitory action of zinc on alcohol-induced liver damage and activation of inflammation was associated with zinc suppression of alcohol-induced intestinal permeability changes. These results thus demonstrate that zinc prevention of increased intestinal permeability is importantly involved in the inhibition of acute ethanol-induced liver damage in mice.
Acute ethanol exposure causes significant damage in the livers of mice with common pathological changes observed including microvesicular steatosis, apoptosis, necrosis, and inflammation (Zhou et al., 2002; Zhou et al., 2001). Importantly, increasing evidence implicates that endotoxemia is a primary cofactor in the induction of acute ethanol-induced liver pathogenesis (Enomoto et al., 1998; Rivera et al., 1998). Endotoxins are glycolipid components of the outer wall of gram-negative bacteria that colonize the large and small intestine (Su, 2002). Prior studies have shown that livers from rats acutely exposed to ethanol experienced a transient increase in plasma endotoxin leading to liver damage (Enomoto et al., 1998; Shibayama et al., 1991). Furthermore, sterilization of the intestinal system with antibiotics prior to ethanol challenge leads to a significant decrease in liver damage of rats, implicating the critical involvement of bacterial endotoxins in alcohol-induced liver injury (Adachi et al., 1995).

Endotoxin is cleared from the circulation by Kupffer cells, the resident macrophages of the liver (Saba, 1970). In response to endotoxin, Kupffer cells produce pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), and reactive oxygen species that are toxic to the surrounding parenchyma (Luster et al., 1994; Arthur et al., 1988). Injection of anti-TNF antibodies as well as pentoxifylline, an inhibitor of TNF-α secretion, into animals has been shown to protect the liver (Tracey et al., 1987; Bachmann et al., 1992). In addition, Kupffer cells have been illustrated to play an important role in hypoxia-reoxygenation induced damage of liver parenchyma following acute ethanol exposure through initiating a swift increase in alcohol metabolism (SIAM) via secretion of prostaglandins (Rivera et al., 1998). Inactivation of Kupffer cells with gadolinium chloride (GdCl₃) in vivo leads to a decrease in liver damage and improves mortality following ethanol treatment in experimental animals (Adachi et al., 1994).
Collectively, these data implicate Kupffer cells as an important cofactor in ethanol-induced liver injury.

Under normal physiological conditions, the intestinal mucosal layer allows small antigens and macromolecules to pass through in small quantity (Bode, 1990). However, acute ethanol exposure increases the permeability of the small intestine to larger macromolecules including bacterial endotoxins (Tamai et al., 2000). The specific mechanisms involved in elevation of intestinal permeability to endotoxin during alcohol exposure remain unclear. However, it has been shown that the effects of acute treatment with ethanol on intestinal membrane structure are dose and time-dependent and the damage incurred by different sections of the small intestine appears to be related to proximity to the gastric compartment (Beck and Dinda, 1981).

Previous studies have described that as many as 2.4 x 10^3 bacteria per gram tissue reside in normal rat small intestine (Yi et al., 1999). These intestinal bacteria “turnover” on a daily basis and as a result release cell wall components, creating an “endotoxin rich” environment in the lumen of the gut. Therefore, increased permeability of the intestine may allow excessive levels of endogenous bacterial endotoxin to leak into portal blood flow causing liver injury. The support for this speculation was that rats treated acutely with ethanol experienced an increase in plasma endotoxin and this was a function of increased intestinal absorption from the small intestine (Tamai et al., 2000). Thus, it is imperative that protection of the liver from the deleterious effects of ethanol mediated by endotoxin should be developed at the level of intestinal mucosa. Recent studies showed that dietary supplementation of rats with oats protected the liver from chronic alcohol injury by preventing intestinal leakiness and endotoxemia, however this result was perplexing because intestinal injury was not significantly different in the oats-treated rats challenged with ethanol from the ethanol treated controls.
(Keshavarzian et al., 2001). Our recent studies have shown that zinc supplementation of mice prior to acute ethanol challenge significantly protected the liver from injury (Zhou et al., 2002). This zinc protection was shown to relate to antioxidant action in hepatocytes. However, zinc supplementation has proven to be protective in many intestinal disease processes that involve changes in permeability including chronic inflammatory bowel disease or Crohn’s disease and acute shigellosis (Sturniolo et al., 2001; Alam et al., 1994). Therefore, we hypothesized that zinc supplementation would ameliorate acute ethanol-induced liver injury by preventing an increase in intestinal permeability and subsequent endotoxemia.
Materials and Methods

Animals. Male 129/SvPCJ mice (20-25 g, 8-10 weeks of age, Jackson Laboratory; Bar Harbor, Maine) were used in this study. They were maintained at 22°C with a 12-hour light/dark cycle and had free access to rodent chow and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

Acute Ethanol Challenge. A binge drinking model developed by Carson and Pruett (1996) was followed for acute ethanol challenge. This model was designed to achieve blood alcohol levels that would produce physiological effects comparable to human binge drinking. Animals were divided into four treatment groups (5 mice per group) in a 2 X 2 factorial design (+ / − zinc, + / − ethanol): 1) isocaloric maltose water control, 2) ethanol, 3) zinc, and 4) zinc plus ethanol. In groups 3 and 4, mice were given ZnSO₄ (Sigma, St. Louis, MO) intragastrically at a dose of 5 mg zinc ion/kg body weight, in 12-hour intervals for 24 h. In groups 1 and 2, mice were given sterile saline intragastrically as a vehicle control. Mice were fasted for 16 hours and mice in groups 3 and 4 then received the third and final dose of zinc. After one hour, in groups 2 and 4, mice were administered ethanol (30 % w/v) (Aldrich, Milwaukee, WI) in a single oral dose of 6 g/kg body weight by gavage and group 1 and 3 mice received isocaloric maltose water by gavage on the same schedule. The time of necropsy for determination of plasma endotoxin levels and analysis of ileal intestine was 1.5 hours following ethanol or maltose water administration. For examination of liver TNF-α, liver histology, and serum enzymes the time of necropsy was 6 hours following ethanol or maltose water. Mice were anesthetized with sodium pentobarbital (0.05 mg/g body weight) (Abbott Laboratories, North Chicago, IL). Blood was drawn from the
dorsal vena cava, livers were perfused and harvested, and 1.0 cm sections of the duodenum, jejenum and ileum were obtained for analysis. Stored tissues were first flash frozen in liquid nitrogen and then placed in -80°C until analysis.

**Blood alcohol assay.** Serum blood alcohol levels were measured using an alcohol dehydrogenase (ADH) kit (Procedure No. 332-UV, Sigma, St. Louis, MO) according to the manufacturer’s instructions.

**Alanine aminotransferase assay.** Serum alanine aminotransferase (ALT, EC 2.6.1.2.) activity was colorimetrically measured using a Diagnostic kit (Procedure No. 505, Sigma, St. Louis, MO) according to the manufacturer’s instructions.

**Histopathological examination of the liver and intestinal sections.** Liver and intestinal histological slides were prepared as described before (Zhou et al., 2002) and Hematoxylin and Eosin (according to Ehrlich, Fluka, Milwaukee, WI) staining of liver and intestinal sections were observed by light microscopy.

**Plasma Endotoxin assay.** Blood samples from control and treated mice were drawn from the dorsal vena cava via sterile heparinized syringes. Platelet-rich plasma was obtained by centrifuging the whole blood at 300 x g for 15 minutes at 4°C. Plasma samples were diluted 1:10 with sterile nanopure water, mixed by vortex and placed in a 75°C water bath for 10 minutes. Samples were allowed to cool to room temperature for 10 minutes prior to colorimetric assay using the limulus ameobocyte lystate (LAL) kit (Biowhittaker, Walkerville, MD).
Standards and samples were incubated with LAL for 10 minutes at 37°C followed by 6 minutes incubation with colorimetric substrate. The reaction was stopped with 25% acetic acid and the absorbance was read in a microplate reader at 405 nm.

*Isolation of Liver TNF-α.* Liver pieces (0.75-1.0 g) were minced thoroughly in ice-cold RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris base, 0.3 % Triton X-100, 0.03 % sodium dodecyl sulfate, 0.3 % Na-deoxycholate, and 1 % protease inhibitor cocktail, pH 7.4) followed by incubation on ice for 30 minutes. The homogenates were then centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatants were removed to clean tubes and centrifuged again at 15,000 x g for 20 minutes at 4°C. The supernatants of this spin were then used for ELISA assay (kit # KMC3012, Biosource, Camarillo, CA).

*Intestinal Permeability Assay.* To evaluate the effect of ethanol on intestinal permeability, bacterial lipopolysaccharide (LPS, E. coli serotype 0111:B4; Sigma, St. Louis, MO) leakage from the intestine by examining blood levels of LPS after intestinal loading was applied. Mice were loaded with different concentrations of LPS and 30 minutes after the loading, the animals were sacrificed and blood was collected for analysis by plasma endotoxin assay. After the dose-response study, a dose of 1 mg/kg body weight LPS was chosen and given to the mice 1 hour after the treatment with isocaloric maltose water or ethanol and the same procedure above was followed to determine the blood endotoxin levels.

*Statistics.* Data were expressed as mean ± SD (n = 5-8) and analyzed according to a 2 X 2 (zinc versus ethanol) factorial experimental design. After a significant interaction was detected by the
two-way analysis of variance, the significance of the main effects was further determined. The level of significance was considered at $P < 0.05$. 
Results

Blood Alcohol Level (BAL)

At 1.5 h after ethanol administration by gavage, the BALs in ethanol treated mice were measured as shown in Figure 1. Alcohol treatment significantly increased BALs and zinc pretreatment did not change this elevation. This result thus indicates that zinc pretreatment did not affect alcohol absorption and that any observed protective effects of zinc in the liver and small intestine were not due to a decreased level of exposure to ethanol.

Inhibition of Acute Ethanol-Induced Liver Injury by Zinc Pretreatment

Oral administration of alcohol caused marked liver injury as examined by serum markers of liver damage and hepatic histopathological changes. As shown in Figure 2, acute ethanol exposure caused a 4-fold increase in the levels of serum alanine aminotransferase (ALT) as compared to control animals. Pretreatment of mice with zinc prevented the elevation of serum ALT, thus indicating the protective effect of zinc on acute ethanol-induced liver injury. The most common histopathological change observed in the liver following acute ethanol treatment is microvesicular steatosis as seen by the engorgement of hepatocytes with fatty vesicles in the cytosolic compartment (Figure 3C). In addition, there was mild necrosis found in the livers of acute ethanol-treated mice, which is characterized as enlarged hepatocytes with faint or absent nuclei (Figure 3C). The livers of control and zinc-treated mice appeared healthy with no observable anomalies (Figure 3A and 3B). Zinc pretreatment significantly inhibited the steatosis and necrosis in the liver as seen by the diminished fatty infiltration of hepatocytes and no observable necrosis (Figure 3D).
Inhibition of Acute Ethanol-Induced Liver TNF-α Production

Acute ethanol exposure elicited a 4-fold increase in liver TNF-α levels as compared to control animals (Figure 4). Zinc-treated animals had levels of liver TNF-α approximately equal to that of untreated controls and mice treated with zinc prior to acute ethanol exhibited significant inhibition ($p<0.05$) to the increase in the liver TNF-α levels (Figure 3).

Inhibition of Acute Ethanol-Induced Endotoxemia

As illustrated in Figure 5, acute ethanol exposure caused a 6-fold increase in the level of plasma endotoxin compared to control mice. Zinc pretreatment significantly ($p<0.01$) inhibited this ethanol-induced effect where the concentration of plasma endotoxin was only slightly elevated compared to zinc-treated control animals.

Prevention of Acute Ethanol-Induced Histopathological Changes in the Intestine by Zinc Pretreatment

Acute ethanol exposure caused significant injury to the ileal small intestine of mice. There was severe injury to the mucosal lining with breaches in the epithelial layer of the villi (Figure 6C). In addition, submucosal blebbing and ulceration of villi were observed (Figure 6C). In contrast, zinc pretreated mice suffered no observable alterations in the ileal small intestine following acute ethanol challenge (Figure 6D). The villi appeared uniform with no blebbing or loss of epithelial cells.
Inhibition of Acute Ethanol-Induced Intestinal Permeability Changes by Zinc Pretreatment

Permeability alterations of mouse intestine due to ethanol were determined by measuring LPS leakage from the intestine 30 min after intragastric loading of exogenous LPS. To define an optimal dose of LPS for the test, different LPS concentrations of 1, 2, 5, and 10 mg/kg were loaded intragastrically. The results showed that an LPS dose of 2 mg/kg or less produced blood levels of endotoxin that were approximately equal to that observed in the control animals. However, LPS doses above 5 mg/kg produced a 5 to 6-fold increase in the detectable blood endotoxin levels. Thus a dose of 1 mg/kg was chosen to determine the effect of ethanol on intestinal permeability. As shown in Figure 7, acute ethanol exposure caused a significant increase (p<0.01) in the levels of plasma endotoxin compared to control mice. Mice treated acutely with ethanol and subsequently loaded with exogenous LPS showed a significant further increase in plasma endotoxin compared to mice administered acute ethanol alone (Figure 7).
Discussion

Previous studies have suggested that dietary supplementation with food products, vitamins, minerals or antioxidants can have protective effects against alcohol-induced liver damage (Lieber, 2000). However, the action sites of the protection have not been fully understood. In this study we found that acute ethanol exposure induced a significant damage to the small intestine in mice, thereby increasing intestinal permeability. This phenomenon was associated with endotoxemia and pathogenesis in the liver. Zinc pretreatment prevented intestinal damage and permeability alterations and this effect was associated with the inhibition of liver injury. Collectively, these results illustrate that zinc acts as a potent inhibitor of acute ethanol-induced liver damage and that this protection is associated with zinc preservation of the barrier function of the small intestine.

Acute ethanol-induced liver injury has been characterized in mice and the pathological changes reported are similar to that of chronic alcohol fed animals, such as steatosis and necrosis (Zhou et al., 2002). When hepatocytes are damaged via necrosis, the cell membrane is compromised allowing release of cytosolic proteins and enzymes such as alanine aminotransferases (ALT) into the circulation. In this study, acute ethanol exposure caused a significant increase in serum ALT levels indicating necrotic damage to the parenchyma and zinc pretreatment inhibited this ethanol-induced injury. Furthermore, morphological analysis of liver sections from acute ethanol-treated mice confirmed the presence of necrotic damage and fat accumulation in hepatocytes. The livers of mice pretreated with zinc had diminished necrosis and decreased microvesicular steatosis. Necrosis has been reported to be associated with inflammation of the liver, therefore zinc protection might relate to its modulation of alcohol-induced inflammatory response of the liver.
Accumulating evidence supports the involvement of Kupffer cells in the pathogenesis of alcohol-induced liver injury (Adachi et al., 1994). Kupffer cells interact with circulating antigens such as endotoxins through their membrane receptors and thus are activated (Decker, 1990). Activated Kupffer cells release various cytokines such as TNF-\(\alpha\) and interleukins, as well as prostaglandins and reactive oxygen radicals (Enomoto et al., 1998; Luster et al., 1994; Martinez et al., 1992). Previous studies have illustrated that alcohol administration increases circulating TNF-\(\alpha\) levels and that down-regulating TNF-\(\alpha\) expression or administering anti-TNF-\(\alpha\) antibodies attenuated alcohol-induced liver injury (Kamimura and Tsukamoto, 1995; Iimuro et al., 1997; Honchel et al., 1990). It is well known that hepatocytes are rich in high-affinity, low-capacity tumor necrosis factor receptors (TNFR’s) rendering them highly sensitive to TNF-\(\alpha\) (Deaciuc et al., 1995). Therefore TNF-\(\alpha\) secreted by activated Kupffer cells has a significant direct toxic effect on the surrounding parenchyma. In this study, we observed a significant increase in the liver TNF-\(\alpha\) levels 6 hours after ethanol exposure and this increase was significantly depressed in the zinc-pretreated animals. This result suggested that zinc prevention of a Kupffer cell-mediated inflammatory response is critical in protecting the liver from acute ethanol-induced injury.

Rats treated acutely with ethanol have been shown to experience a transient increase in plasma endotoxin with maximal levels observed one hour following administration (Enomoto et al., 1998). Adachi et al. (1995) showed that sterilization of the intestine of rats with antibiotics caused a decrease in plasma endotoxin and this result was associated with a significant inhibition of alcohol-induced liver injury. In rodent models of lethal shock, the mortality rate of mice challenged with an intraperitoneal injection of endotoxin was 100% and this effect was almost completely abolished when mice were pretreated with zinc and 97% of the animals survived.
To determine whether zinc protection from acute alcohol-induced liver injury is through inhibition of endotoxemia, we measured plasma endotoxin 1.5 hr after ethanol exposure, which has been shown previously to be in the peak time range for alcohol-induced endotoxemia (Rivera et al., 1998). We observed a 6-fold increase in plasma endotoxin of mice treated acutely with ethanol as compared to control mice and zinc pretreatment almost completely blocked this ethanol-induced effect. It should be noted that we observed basal levels of plasma endotoxin in control (13.1 ± 6.6 pg/ml) and zinc-treated (14.5 ± 5.8 pg/ml) mice.

Many previous studies reported near zero or undetectable levels of endotoxin in control animals (Rivera et al., 1998; Keshavarzian et al., 2001). However, it has been discussed in a recent study that there are a number of reasons for high baseline LPS levels in animal models of alcoholic liver disease in control animals (Mathurin et al., 2000). We were not able to define specific causes for the high basal levels of LPS in our control animals, although this stable high basal level of LPS has been consistently observed in our studies.

Acute ethanol-induced endotoxemia is hypothesized to be a result of increased intestinal permeability to endotoxin. Reports of ethanol-induced increases in intestinal permeability in the absence of morphological damage are common (Keshavarzian et al., 1994; Worthington et al., 1978). However, our work differed from many of the earlier acute ethanol studies. First, we looked at intestinal morphology 1.5 hours after acute ethanol administration when the levels of plasma endotoxin measured were maximal. In contrast, the time points for harvesting intestinal tissue for histochemical analysis in other studies were from several hours to several days following acute ethanol exposure (Keshavarzian et al., 1994; Worthington et al., 1978). It is imperative to examine tissue before repair has taken place and clearly the intestine is capable of restoring both morphological alterations and decreases in barrier function during recovery from
alcohol challenge (Tamai et al., 2000; Millan et al., 1980). Ileal intestine from mice treated acutely with ethanol in our study suffered gross morphological anomalies where many intestinal villi had breaches in the epithelial layer. Zinc pretreatment profoundly protected the ileal intestine from this acute ethanol-induced morphological phenomenon. Examination of zinc and ethanol treated ileal sections showed normal villi with intact epithelial cell layers and no detectable abnormalities.

Secondly, determination of intestinal permeability in various ethanol studies ex vivo and in vivo has involved the use of macromolecules such as horseradish peroxidase (HRP), lactulose, mannitol or $^{51}$Chromium-ethylenediaminetetraacetic acid ($^{51}$Cr-EDTA) (Keshavarzian et al., 2001; Keshavarzian et al., 1994; Draper et al., 1983; Bjarnason et al., 1984). In this study we employed intragastric administration of exogenous LPS for determination of intestinal permeability because increased permeability to molecular probes such as HRP, EDTA or sugars in the small intestine is not entirely indicative of increased permeability to endotoxins. A complicating issue regarding the use of LPS as a marker of increased intestinal permeability is that LPS may cause increases in permeability by itself (Garcia et al., 2001). We examined dose-response effects of LPS on intestinal permeability and found that high doses of LPS ($\geq 5$ mg/kg body weight) increased plasma endotoxin levels significantly. According to our data, administration of 1 mg/kg LPS alone did not cause an increase in intestinal permeability compared to untreated controls. More importantly, acute ethanol exposure caused significant increases in intestinal permeability to endotoxin as illustrated by the increased level of plasma endotoxin detected in mice treated acutely with ethanol and loaded with LPS as compared to animals treated with ethanol alone. This result suggests that the endotoxemia observed in our work was a result of acute ethanol-induced increase in intestinal permeability to endotoxin and
that zinc inhibition of circulating plasma endotoxin is due to preservation of intestinal barrier function.

Although the results of this study provide evidence that zinc supplementation had potent inhibitory effects on acute ethanol-induced liver injury via prevention of intestinal damage, there are questions and limitations that need to be addressed pertaining to the mechanisms in which zinc provides this protection and particularly if these effects are due to zinc action alone. In previous studies, we have shown that zinc supplementation ameliorates cell damage in the liver parenchyma and this was associated with inhibition of oxidative stress (Zhou et al., 2002). More recently, we found that acute ethanol-induced hepatocyte cell death mediated through the Fas (CD95/Apo-1)/Fas ligand pathway was significantly inhibited by zinc pretreatment (Lambert et al., in press). In addition, zinc is well known to be protective in cells of the macrophage-monocyte lineage, though the mechanism is still not clear (Brown and Carter, 1990). Therefore, although we observed a correlation between zinc’s protective effect against TNF-α production and the inhibition of acute alcohol-induced increases in endotoxin absorption at the level of the intestine, we cannot rule out direct protection of Kupffer cells by zinc. Intestinal absorption of zinc is primarily dependent upon metallothionein and this zinc binding protein has been shown in many experimental systems to have significant protective effects (Takano et al., 2000; Cunningham-Rundles et al., 1999). Further studies are required to delineate if the inhibitory actions by zinc at the intestinal mucosal layer are independent of metallothionein. Lastly, we cannot exclude the possibility that zinc administered intragastrically might have a protective effect on intestinal microflora leading to a decrease in endotoxin release in the lumen of the intestine during acute ethanol challenge.
In conclusion, this study demonstrated that zinc is directly involved in inhibition of acute alcohol-induced liver injury and this protection entails prevention of endotoxemia through preservation of intestinal barrier function. The significance of this finding is that dietary zinc supplementation inhibits acute ethanol-induced liver injury at different levels and that an important site of zinc protection may be in the intestine.
Footnotes

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References


Brown D and Carter DM (1990) Physiological and pharmacological effects of zinc on


Figure legends

Figure 1. Blood alcohol levels measured from sera obtained 1.5 h after intragastric administration of 6 g/kg alcohol (EtOH, n=9). Controls were treated by the same route with isocaloric maltose water (Con, n=7). Some mice were treated with 5 mg/kg ZnSO₄ three times in 12-h intervals and administrated with isocaloric maltose water one hour after the last dose of zinc (Zn, n=7) and others were pretreated with zinc followed by ethanol challenge one hour after the last zinc administration (Zn+EtOH, n=9). Results are means ± SD. *Significantly different from control (Con) group.

Figure 2. Serum ALT levels 6 h after intragastric administration of alcohol. These mice were administered 6 g/kg alcohol by gavage (EtOH, n=8) and an equivalent volume of isocaloric maltose water was given to controls (Con, n=6). Some mice were treated with 5 mg/kg ZnSO₄ three times in 12-h intervals followed by isocaloric maltose water treatment as described for Figure 1 (Zn, n=6) and others were pretreated with zinc followed by ethanol challenge one hour after the last zinc administration (Zn+EtOH, n=8). The serum ALT activities were determined by colorimetric assay. Results are means ± SD. *Significantly different from control (Con) group.

Figure 3. Acute ethanol-induced histopathological changes in the livers 6 h after intragastric administration of 6g/kg ethanol: control (A), zinc (B), ethanol (C), and zinc + ethanol (D); (E and F are higher magnifications of C and D, respectively). The treatment protocol was as the same as that described for Figure 1. The major histopathological change induced by ethanol in mouse liver was microvesicular steatosis (arrows). In addition, ethanol administration caused
hepatocyte enlargement with nuclear dissolution (indicative of necrosis, arrowheads). Zinc pretreatment suppressed these ethanol-induced alterations. CV, central vein. H&E staining; (A-D) magnification, X 260; E and F, X 520.

Figure 4. Intrahepatic TNF-α levels 6 h after intragastric administration of ethanol. The animals were subjected to the same treatment protocol as described for Figure 1. (Con, n=6), control; (EtOH, n=8), ethanol; (Zn, n=6), zinc; (Zn+EtOH, n=8), zinc plus ethanol. Liver TNF-α levels were determined by ELISA assay. Results are means ± SD. *Significantly different from control (Con) group and †significantly different from ethanol-treated (EtOH) group.

Figure 5. Plasma endotoxin levels 1.5 h after intragastric administration of ethanol (EtOH, n=9). Controls were treated by the same route with isocaloric maltose water (Con, n=7). Some mice were treated with 5 mg/kg ZnSO₄ three times in 12-h intervals and administrated with isocaloric maltose water one hour after the last dose of zinc (Zn, n=7) and others were pretreated with zinc followed by ethanol challenge one hour after the last zinc administration (Zn+EtOH, n=9). Results are means ± SD. *Significantly different from control (Con) group.

Figure 6. Acute ethanol-induced histopathological changes in the small intestine 1.5 h after intragastric administration of 6g/kg ethanol: control (A), zinc (B), ethanol (C), and zinc + ethanol (D). The animals were treated following the same protocol described for Figure 1. Ethanol treatment caused loss of epithelial cells of ileal villi (arrows) and ulceration and blebbing of the lamina propria. Zinc pretreatment inhibited these ethanol-induced morphological anomalies. H&E staining; magnification, X260.
Figure 7. Plasma endotoxin levels in LPS challenged mice. Mice were treated intragastrically with either isocaloric maltose water (Con, n=6), lipopolysaccharide (LPS, n=6), ethanol (EtOH, n=8), or both LPS and EtOH (EtOH+LPS, n=8). The animals were administered 6 g/kg alcohol by gavage or the same volume of isocaloric maltose water. One hour later, mice in the (LPS) and (EtOH+LPS) groups received 1 mg/kg bacterial lipopolysaccharide (LPS) by intragastric gavage. Thirty minutes after LPS administration, plasma endotoxin concentrations were determined by limulus amoebocyte lysate (LAL) colorimetric assay. Results are means ± SD. *Significantly different from control (Con) group and †significantly different from ethanol-treated (EtOH) group.
Figure 1

![Graph depicting % Blood alcohol (w/v) for different conditions: Con, EtOH, Zn, and Zn+EtOH. The graph shows that EtOH and Zn+EtOH have significantly higher blood alcohol levels compared to Con and Zn, indicated by asterisks.](image-url)
Figure 3

A

B

C

D

E

F
Figure 4

Liver TNF-alpha (pg/mL)

Con  EtOH  Zn  Zn+EtOH

*  †

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Figure 7

Plasma Endotoxin (pg/mL)

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<th>Condition</th>
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* Significant difference compared to Con
† Significant difference compared to LPS

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