Metallothionein Protection against Alcoholic Liver Injury through Inhibition of Oxidative Stress

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Antioxidants are likely potential pharmaceutical agents for the treatment of alcoholic liver disease. Metallothionein (MT) is a cysteine-rich protein and functions as an antioxidant. This study was designed to determine whether MT confers resistance to acute alcohol-induced hepatotoxicity and to explore the mechanistic link between oxidative stress and alcoholic liver injury. MT-overexpressing transgenic and wild-type mice were administrated three gastric doses of alcohol at 5 g/kg. Liver injury, oxidative stress, and ethanol metabolism-associated changes were determined. Acute ethanol administration in the wild-type mice caused prominent microvesicular steatosis, along with necrosis and elevation of serum alanine aminotransferase. Ultrastructural changes of the hepatocytes include glycogen and fat accumulation, organelle abnormality, and focal cytoplasmic degeneration. This acute alcohol hepatotoxicity was significantly inhibited in the MT-transgenic mice. Furthermore, ethanol treatment decreased hepatic-reduced glutathione, but increased oxidized glutathione along with lipid peroxidation, protein oxidation, and superoxide generation in the wild-type mice. This hepatic oxidative stress was significantly suppressed in the MT-transgenic mice. However, MT did not affect the ethanol metabolism-associated decrease in NAD^+/NADH ratio or increase in cytochrome P450 2E1. In conclusion, MT is an effective agent in cytoprotection against alcohol-induced liver injury, and hepatic protection by MT is likely through inhibition of alcohol-induced oxidative stress.

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Key words: alcohol; hepatotoxicity; metallothionein; oxidative stress

Alcohol abuse is a major health problem worldwide. Alcoholic liver disease is responsible for 15%–30% of all admissions in many general hospitals (1). The overall economic cost of alcohol abuse in the United States was estimated to be $184.6 billion in 1998, rising 25% from 1992 (2). Although important progress has been made in understanding the pathogenesis of alcoholic liver disease, current therapies for this disease are not effective. Novel therapeutic approaches such as utilizing agents that successfully correct the fundamental cellular disturbances resulting from excessive alcohol consumption are attractive.

There is increasing evidence that oxidative stress plays an important etiologic role in the development of alcoholic liver disease. Alcohol administration has been found to cause accumulation of reactive oxygen species, including superoxide, hydroxyl radical, and hydrogen peroxide (3). Reactive oxygen species, in turn, cause lipid peroxidation of cellular membranes, and protein and DNA oxidation, which results in hepatocyte injury (4–6).

Based on the hypothesis that oxidative stress occurs only when the antioxidant capacity is insufficient to cope with the generation of pro-oxidants (3), many studies have focused on the ethanol-associated changes in the liver antioxidants. One of the most prominent defense systems in the liver is the presence of reduced glutathione (GSH). Besides serving as a substrate for glutathione-related enzymes, GSH acts as a free radical scavenger, a regenerator of α-tocopherol, and plays an important role in the maintenance of protein sulfhydryl groups. Most studies have reported that acute ethanol administration decreases hepatic GSH content (7–9). The decrease in GSH has been related to an enhanced oxidation of GSH to oxidized glutathione (GSSG) as a consequence of increased generation of reactive oxygen species (10). Restoration of GSH has been shown to inhibit ethanol-induced liver injury (11, 12). These results suggest that increasing hepatic antioxidant defense is likely a potential therapy for alcoholic liver disease.

One potential antioxidant is metallothionein (MT), a highly conserved, low-molecular-weight, thiol-rich protein

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(13, 14). MT shares an important similarity with GSH due to the fact that one-third of their amino acids are cysteines. The thiol groups of GSH and MT are the main source of thiol in cells. Importantly, the thiol groups in MT are preferential attacking targets for free radicals compared with the other sulfhydryl residues such as that from GSH and protein fractions (15). Kinetic study *in vitro* also demonstrated that MT was 38.5-fold more potent than glutathione on the molar basis in preventing hydroxyl radical-generated DNA degradation (16). Experimental depletion of GSH in hepatocytes has been shown to trigger MT expression and thereby to suppress hepatotoxicity (17, 18). These studies indicate that MT is an important component in the antioxidant defense system in the cells.

To study the function of MT *in vivo*, a MT-overexpressing transgenic (MT-TG) mouse model has been produced (19). In the MT-TG mice, hepatic MT was elevated about 10-fold, with no changes in other antioxidant activities (20). This model thus serves as a valuable tool to study whether increased antioxidant capacity constitutively can prevent alcohol-induced hepatic injury and to study the role of MT in hepatic protection against alcohol toxicity. This study was undertaken to determine the effect of MT on acute alcohol-induced hepatotoxicity and to explore the mechanistic link between oxidative stress and alcoholic liver injury by using the MT-TG mouse model.

Materials and Methods

Animals. MT-TG mice and the wild-type C57BL/6 controls were obtained from The Jackson Laboratory (Bar Harbor, ME). MT-TG mice overexpress MT-I, the predominant form of MT in the liver (22). The mice were housed in the animal quarters at the University of Louisville Research Resources Center. They were maintained at 22°C with a 12:12-hr light:dark cycle and they had free access to rodent chow and tap water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which was certified by the American Association of Accreditation of Laboratory Animal Care.

Ethanol Administration. A binge drinking mouse model developed by Carson and Pruett (21) was followed for ethanol challenge. This model was designed to achieve blood alcohol levels, behavioral effects, and physiological changes comparable with human binge drinking. MT-TG as well as wild-type mice at the age of 9 weeks were divided into control and ethanol treatment groups, with 32 mice in each group. Three doses of 25% (w/v) ethanol were administered at 5 g/kg body weight by gavage every 12 hr. Control mice received isocalorical maltose solution. At 4 hr after the last dosing, the mice were anesthetized with sodium pentobarbital (0.05 mg/g body weight). Blood was drawn from the dorsal vena cava, and sera were obtained by centrifugation using a serum separator tube.

MT Assay. Tissue MT concentrations were determined by a cadmium-hemoglobin affinity assay. Briefly, liver tissues were homogenized in 4 volumes of 10 mM Tris-HCl buffer, pH 7.4, at 4°C. After centrifugation of the homogenate at 10,000*g* for 15 min, 200 μl of supernatant was transferred to microtubes for MT analysis as described previously (22).

Alanine Aminotransferase Assay. Serum alanine aminotransferase (ALT, EC 2.6.1.2.) activity was colorimetrically measured using a Diagnostic kit (Procedure No. 505, Sigma Chemical Co., St. Louis, MO) according to the instruction provided.

Histopathological Examination. Liver tissues were cut into ~3-mm-thick slices and fixed with 10% neutral formalin. The tissue slices were embedded in paraplast. Tissue sections of 5 μm were stained by hematoxylin and eosin, and were observed with a Nikon Eclipse E400 light microscope.

Ultrastructural Changes. To observe ethanol-induced ultrastructural changes by conventional electron microscopy, the livers of all of the experimental animals were fixed *in situ* by vascular perfusion with Karnovsky's fixative (2.5% paraformaldehyde and 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4), and were post-fixed in 1% osmium tetraoxide. Ultrathin sections were stained by uranyl acetate and lead citrate, and were observed with a Philips transmission electron microscope.

Determination of GSH and GSSG Concentrations. Liver GSH and GSSG concentrations were determined by the glutathione-disulfide reductase and 5'5-dithiobis (2-nitrobenzoic acid) recycling assay (25). Briefly, liver tissue was homogenized with 5% sulfosalicylic acid and was centrifuged at 10,000*g* for 5 min. The supernatant was divided into two aliquots. One aliquot was directly used to measure total glutathione and the other aliquot was first treated with 2-vinylpyridine to block the GSH, followed by measuring the GSSG. The GSH value was determined by subtracting GSSG from total glutathione.

Lipid Peroxidation Assay. Hepatic lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously (26). Liver tissue was homogenized in 9 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 180 mM KCl, 10 mM EDTA, and 0.02% butylated hydroxytoluene. To 0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, 1.5 ml of 0.9% thiobarbituric acid, and 0.6 ml of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95°C for 1 hr. After cooling on ice, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After centrifugation at 10,000*g* for 10 min, the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,5,5-tetraethoxypropane as a standard.

Measurement of Oxidized Protein. The oxidized protein was assessed by determination of carbonyl concentration (27). Liver tissue was homogenized in 9 volumes of 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, and protein inhibitors (20 μg/ml leupeptin, 10

μg/ml pepstatin A, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl). To 1.0 ml of homogenate (approximately 5 mg of protein), 4 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl was added; or 4 ml of 2 N HCl was added as a blank. The mixture was incubated for 1 hr at room temperature. The protein was precipitated with an equal volume of 20% trichloroacetic acid and was washed three times with ethanol/ethylacetate (1:1, v/v). The final precipitate was dissolved in 2 ml of 6 *M* guanidine hydrochloride (pH 2.3) and insoluble debris was removed by centrifugation. The DNPH derivatives were measured at 360 nm. The concentration of carbonyl groups was calculated by using an absorbance coefficient 22 mM^{-1} cm^{-1}.

Determination of NAD⁺/NADH Ratio. Cytoplasmic NAD^+/NADH ratio in the liver was estimated by enzymatically measuring lactate and pyruvate concentrations using Diagnostic kits (Sigma). Briefly, liver tissue was homogenized in ice-cold 6% perchloric acid and was centrifuged at 25,000*g*. The supernatant was then neutralized with 5 *M* K_2CO_3 and was used for lactate and pyruvate assays. The NAD^+/NADH ratio was calculated using the following equation: $NAD^+/NADH = [pyruvate]/[lactate] \times 1/K_{eq}$ LDH (23).

Determination of Cytochrome P450 2E1 (CYP2E1) Activity. Hydroxylation of *p*-nitrophenol to 4-nitrocatechol, a reaction catalyzed specifically by CYP2E1, was determined colorimetrically as described by Koop (24). Liver tissue was homogenized in 0.15 *M* KCl and was spun at 10,000*g* for 30 min. Microsomes were isolated by further centrifugation at 105,000*g* for 60 min. For the assay, 300 μg of microsomal protein was incubated for 5 min at 37°C, and absorbance at 535 nm was measured with 4-nitrocatechol as a standard. The CYP2E1-catalized *p*-nitrophenol hydroxylation was expressed as nanomoles of product formed per minute per milligram of microsomal protein.

Detection of Superoxide Anion. Accumulation of superoxide anion in the liver was quantified using a cytochrome reduction assay (28). Liver tissue was homogenized and centrifuged at 800*g*. The supernatant was incubated in the presence of 30 μ*M* succinylated cytochrome *c* and 1 mM NADPH. The change in absorbance at 550 nm was measured. The difference in the amount of reduced succinylated cytochrome *c* in the presence or absence of 0.2 mg/ml superoxide dismutase was used to estimate the amount of superoxide anion by employing an absorbance coefficient 21.1 M^{-1} cm^{-1}.

Statistics. The data are expressed as mean ± SD (n = 5–7). Differences were tested by Student t test and were considered to be significant when $P < 0.05$.

Results

Liver MT Concentration. As shown in Figure 1, the MT concentration in the liver of MT-TG mice was about 10 times as much as that in wild-type mice. Ethanol administration induced a significant increase in MT concentrations in both MT-TG and wild-type mice. However, the MT concentration in the MT-TG mouse liver remained about eight times higher than in the wild type after ethanol administration.

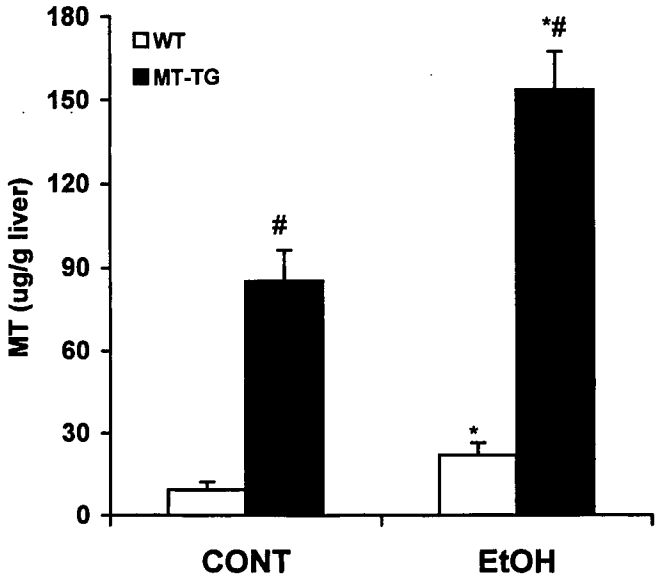

Figure 1. Hepatic MT concentrations of MT-TG and wild-type mice after acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and MT concentrations were determined by a cadmium-hemoglobin affinity assay. Results are means ± SD (n = 5–7). *, Significantly different between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

Effects of MT on Serum ALT Level and Histopathological Changes. Ethanol administration caused elevation of serum ALT in the wild-type mice, but not in the MT-TG mice (Fig. 2). The prominent histopathological change induced by ethanol in the wild-type mouse liver was microvesicular steatosis; however, hepatocyte necrosis was also observed as recognized by cell enlargement and nuclear dissolution (Fig. 3). In comparison, ethanol administration only caused minor microvesicular steatosis in the MT-TG mouse liver.

Prevention by MT of the Ultrastructural Pathologic Changes of Hepatocytes. In comparison with normal controls (Fig. 4, A and B), ethanol administration caused serious ultrastructural changes in the hepatocytes of the wild-type mice (Fig. 4, C and E). The prominent changes were glycogen and fat accumulation, organelle abnormality, and focal cytoplasmic degeneration. One to several large fat droplets, surrounded by glycogen granules, could be found in the cytoplasm. Mitochondrial abnormalities were observed, including variations in size and shape and the reduction or disappearance of cristae. The rough endoplasmic reticulum was disarranged and apparently degenerated. Focal cytoplasmic degeneration was often found, and the condensation of chromatin was also occasionally observed. In the MT-TG mice, ethanol administration caused much less glycogen and fat accumulation and did not change the ultrastructure of the organelles (Fig. 4, D and F).

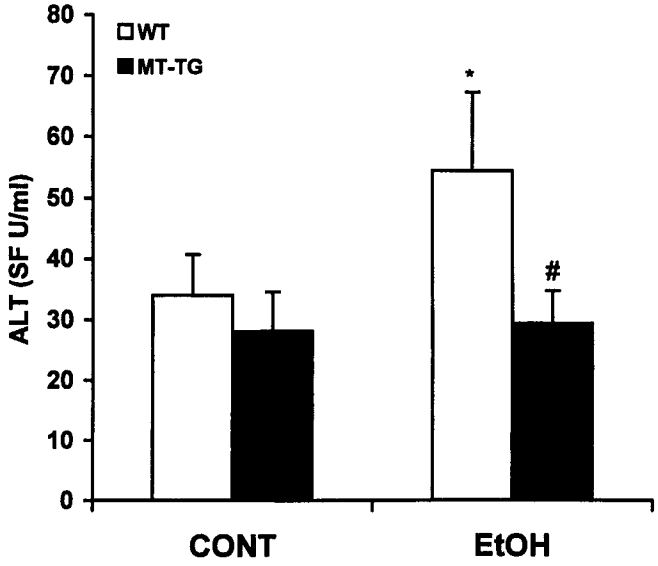

Figure 2. Changes in the serum ALT level induced by acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and ALT activities were colorimetrically measured using a Sigma Diagnostic kit. Results are means ± SD (n = 5–7). *, Significantly different between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

Effects of MT on Hepatic GSH and GSSG Concentrations. There was no significant difference in either hepatic GSH or GSSG concentrations between the MT-TG and wild-type mice, although the MT-TG mice showed relatively lower GSSG concentrations (Fig. 5). In response to ethanol exposure, hepatic GSH concentrations were significantly decreased in the wild-type mice, but not in the MT-TG mice. Ethanol administration also caused a significant increase in GSSG concentration in the liver of wild-type mice.

Inhibition by MT of Ethanol-Induced Hepatic Oxidative Changes. Ethanol-induced hepatic oxidation was assessed by measuring the extent of TBARS and protein carbonyl content. In the wild-type mice, ethanol administration caused a 17-fold increase in the TBARS content in the liver. In the MT-TG mice, the ethanol-induced elevation of TBARS was significantly inhibited, being only one-third of that in the wild-type mice (Fig. 6). The protein carbonyl content was elevated 2.7-fold in the wild-type mouse liver by ethanol treatment. However, there was no significant increase in the protein carbonyl content in the ethanol-treated MT-TG mouse liver (Fig. 7).

Changes in NAD^+/NADH Ratio and CYP2E1 Activity. To determine whether MT influences ethanol metabolism, hepatic NAD^+/NADH ratio and CYP2E1 activity were measured. As shown in Table I, cytoplasmic NAD^+/NADH ratio in the liver was significantly decreased by ethanol treatment, but no significant difference was found between the wild-type and MT-TG mice. Ethanol administration induced about a 2.5-fold increase in hepatic microsomal CYP2E1 activities in both wild-type and MT-TG mice (Table II).

Suppression by MT of Hepatic Superoxide Accumulation. Because MT did not appear to affect ethanol metabolism in the liver as shown above, it is likely to inhibit oxidative stress by ethanol through direct interaction with free radicals. Many studies have shown that MT is a scavenger of superoxide anion (13, 14). Therefore, to elucidate possible mechanisms by which MT inhibits ethanol-induced oxidative stress, the effect of MT on superoxide anion accumulation, which is mainly generated by CYP2E1, was determined. As shown in Figure 8, ethanol administration significantly elevated the superoxide concentration in the wild-type mouse liver. However, this elevation was largely suppressed in the MT-TG liver.

Discussion

The data obtained show that acute ethanol administration in the wild-type mice caused prominent microvesicular steatosis along with necrosis in the liver, which corresponded to the elevation of serum ALT activity. These pathological changes were accompanied with ultrastructural alterations of the hepatocytes, including glycogen and fat accumulation, organelle abnormality, and focal cytoplasmic degeneration. However, this acute alcohol hepatotoxicity was significantly suppressed in the MT-TG mice. In association with the hepatocyte injury, acute alcohol administration induced marked decreases in NAD^+/NADH and GSH/GSSG ratios, and increases in CYP2E1 activity along with lipid peroxidation, protein oxidation, and superoxide accumulation in the wild-type mice. Overexpression of MT in the transgenic mouse liver did not affect ethanol metabolism, but significantly inhibited ethanol-associated oxidative stress.

Although the morphological change is the most direct sign of liver injury, light and electron microscopic observations have been largely ignored in previous studies of acute alcohol hepatotoxicity. In our study, the microvesicular steatosis was shown to be the prominent histopathological change associated with acute ethanol administration. In contrast, the liver injury observed by light microscopy in chronic ethanol administration is characterized by macrovesicular steatosis, inflammation, and necrosis (29, 30). In our study, the ultrastructural changes were observed to occur mainly in the mitochondria of the hepatocytes. However, the prominent subcellular events associated with chronic ethanol administration were the dramatic alterations of the smooth endoplasmic reticulum and mitochondria of the hepatocytes in both animal models and human clinical studies (31, 32). Investigations concerning the functional significance of altered mitochondrial morphology are controversial. Most studies have demonstrated that acute alcohol administration results in alterations of mitochondrial composition and fluidity that would impair the mitochondrial function (33). However, other studies have indicated that the adaptive modification in composition and structure would lead to increased resistance to the disruption of mitochondrial membrane by ethanol (34, 35). However, the

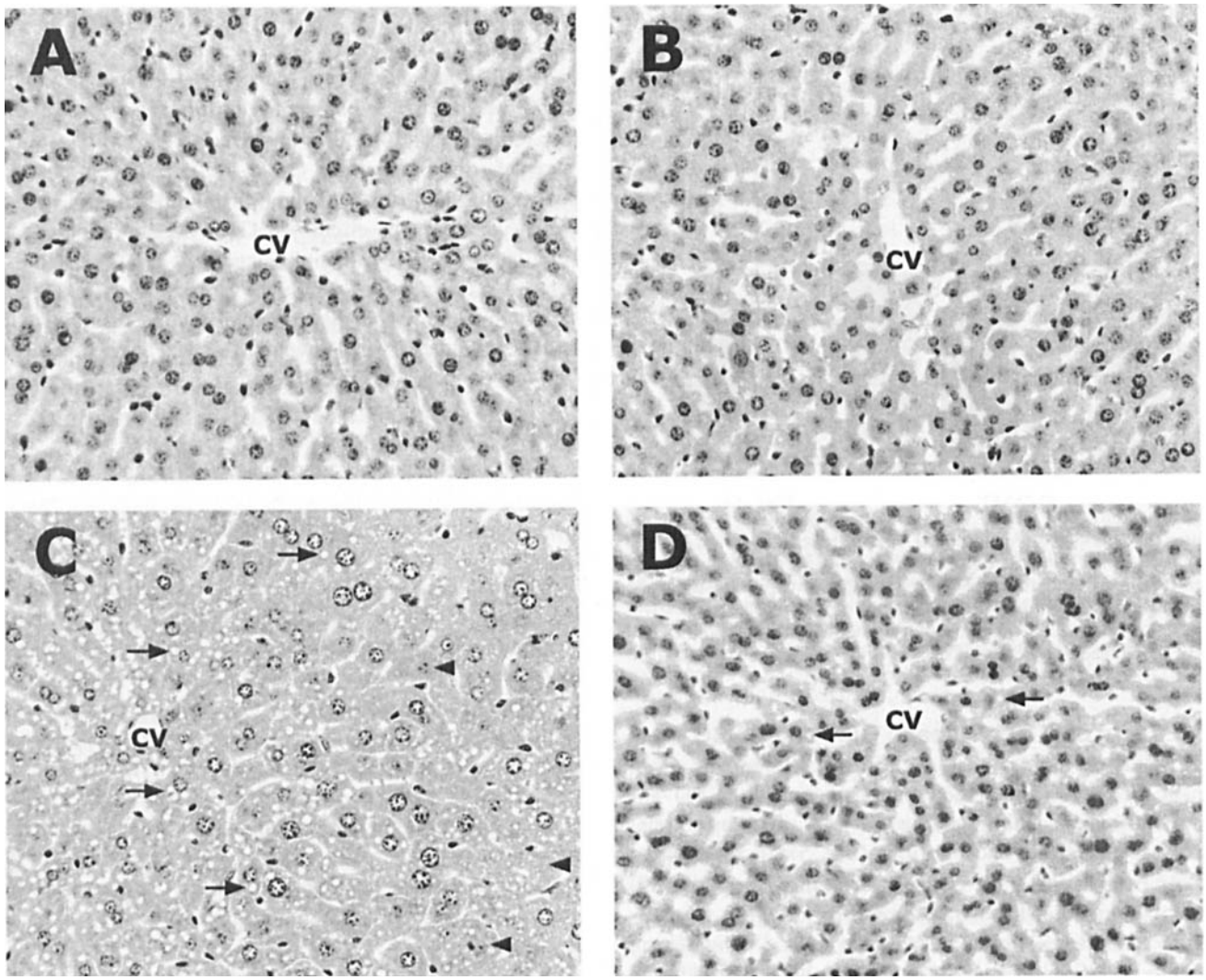

Figure 3. Histopathological changes in the liver induced by acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and liver sections were stained by hematoxylin and eosin staining. (A and B) The normal liver structure in wild-type and MT-TG mice, respectively. Ethanol treatment induced prominent microvesicular steatosis (arrows) along with necrosis (arrowheads) in the liver of wild-type mice (C). The necrotic hepatocytes are characterized by cell enlargement and nuclear dissolution. However, only microvesicular steatosis, which was largely suppressed, was observed in the liver of MT-TG mice (D). CV, central vein. ×260.

results obtained in our study demonstrate that there is coexistence of ethanol-induced changes in mitochondrial appearance (shape and size) and damages to mitochondria (disappearance of cristae). Elevation of MT prevented all these changes and resulted in the maintenance of the integrity of mitochondrial structure.

Oxidative stress, which is defined as an increase in cellular ratio between pro-oxidants and antioxidants, has been demonstrated to mediate the pathogenesis of ethanol-induced liver injury (3). Although the enhanced generation of reactive oxygen species has been constantly reported, published studies of changes in hepatic antioxidant systems after acute ethanol administration are controversial. One of the well-studied antioxidants is GSH. A decrease in cellular GSH content associated with acute ethanol liver injury has been often reported (7–9). However, some studies did not observe significant changes in hepatic GSH and GSSG or the ratio of GSH to GSSG (36, 37). In our study, a significant decrease in hepatic GSH and a significant increase in hepatic GSSG was observed in the wild-type mice, but not in the MT-TG mice. Because the decreased GSH is related to the enhanced oxidation of GSH to GSSG as a consequence of increased accumulation of free radicals (10), the undetectable changes in the hepatic GSH and GSSG in the MT-TG mice most likely resulted from free radical scavenging action of MT. This result suggests that a compensation mechanism may exist between MT and GSH in cellular response to oxidative stress.

Hepatic lipid peroxidation associated with acute ethanol administration, as an indicator of oxidative stress, has been often determined in both animal models and human clinical trials. Excess lipid peroxidation as measured by formation of TBARS or diene conjugates has been found in most studies (3). Masini *et al.* (9) provided convincing evidence that lipid peroxidation induces mitochondrial dysfunction in rats subjected to acute ethanol intoxication. A

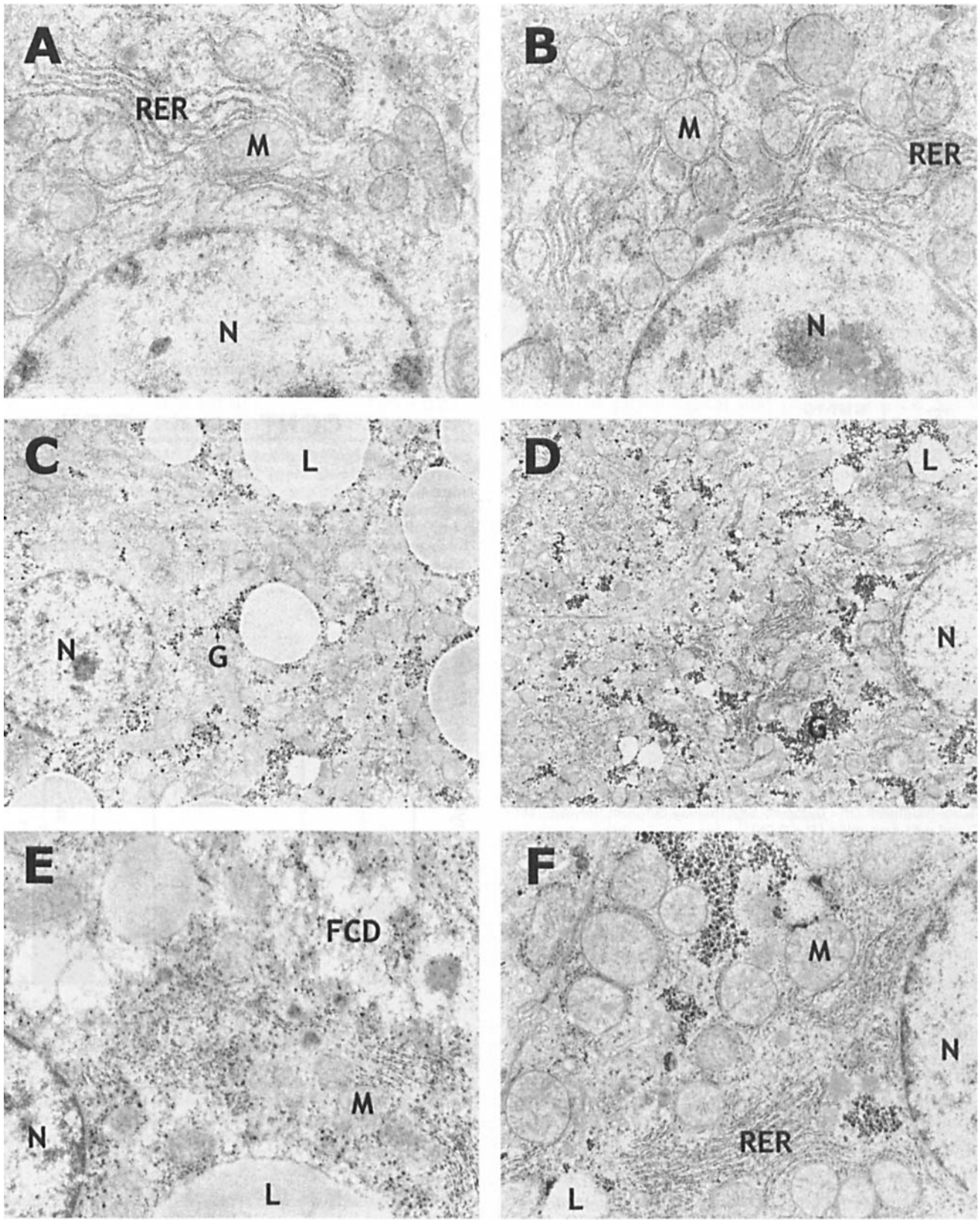

Figure 4. Ultrastructural changes in the hepatocytes induced by acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and liver tissues were processed for conventional electron microscopy. (A and B) Normal ultrastructure of hepatocytes in wild-type and MT-TG mice, respectively. Ethanol treatment caused glycogen and fat accumulation, organelle abnormality, and focal cytoplasmic degeneration (C and E) in the wild-type mice. The ethanol-associated ultrastructural changes were largely inhibited in the MT-TG mice (D and F). M, mitochondria; RER, rough endoplasmic reticulum; L, lipid droplets; G, glycogen; FCD, focal cytoplasmic degeneration. A, B, E, and F, ×11,000; C and D, ×3,300.

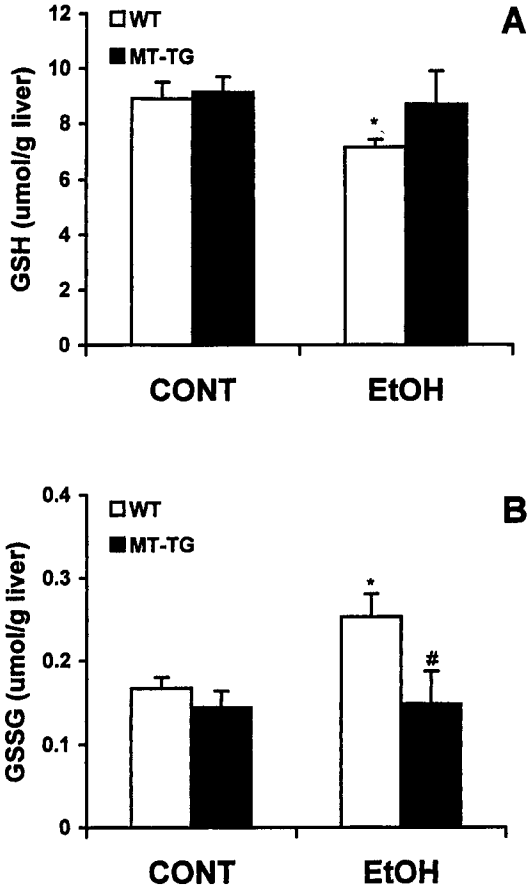

Figure 5. Changes in hepatic GSH and GSSG concentrations induced by acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and GSH and GSSG concentrations in the liver were assayed by the glutathione-disulfide reductase and 5'5-dithiobis (2-nitrobenzoic acid) recycling assay. (A) Hepatic GSH concentrations; (B) Hepatic GSSG concentrations. Results are means ± SD (n = 5–7). *, Significantly different between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

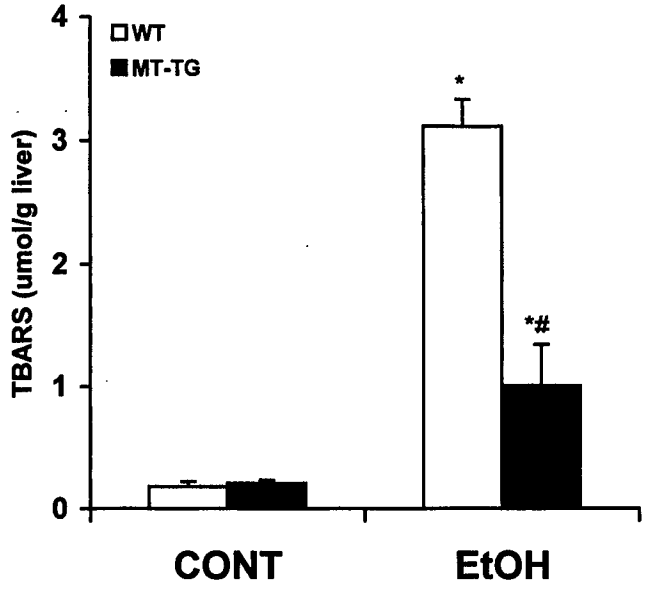

Figure 6. Hepatic lipid peroxidation (TBARS content) induced by acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and hepatic TBARS concentrations were colorimetrically measured using 1,1,5,5-tetraethoxypropane as standard. Results are means ± SD (n = 5–7). *, Significantly different between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

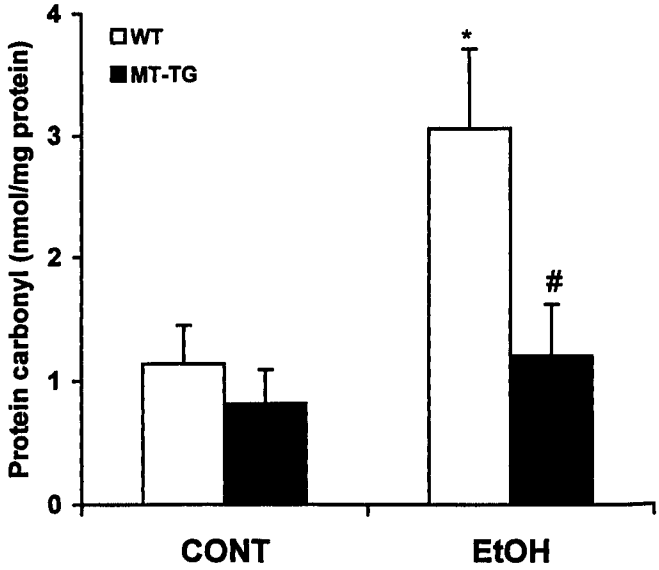

Figure 7. Hepatic protein oxidation (carbonyl content) induced by acute ethanol administration. The carbonyl content was elevated about 2.7-fold in the wild-type mice by ethanol induction, but no significant elevation was found in the MT-TG mice. Results are means ± SD (n = 5–7). *, Significant difference between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

dramatic increase in lipid peroxidation in the wild-type mouse liver after acute alcohol treatment was observed in our study. This increase was significantly inhibited in the MT-TG mice. In agreement with this finding, MT also inhibited alcohol-induced protein oxidation.

An important question is how MT inhibits ethanol-induced oxidative stress in the liver. It has been shown that ethanol metabolism-associated increases in NADH and CYP2E1 are the major contributors to generation of superoxide anion. Ethanol oxidization leads to NADH elevation, and reoxidation of NADH by aldehyde oxidase generates NAD^+ plus superoxide (38). CYP2E1 catalyzes ethanol to acetaldehyde and at the same time reduces dioxygen to a variety of reactive oxygen species, including superoxide (39). Thus, it is important to know whether MT interferes with ethanol metabolism. The results obtained indicate that ethanol increased CYP2E1 activities in the liver of wild-type mice and enhanced the production of NADH. This activation of ethanol metabolism was not diminished in the MT-TG mouse liver. Therefore, MT did not affect alcohol metabolism in the liver.

Elevation of NADH and CYP2E1 in association with ethanol metabolism would lead to increased production of superoxide anion. It has been shown that MT directly interacts with superoxide to prevent the damage induced by

Table I. Effect of Acute Ethanol Treatment on Cytoplasmic $NAD^+/NADH$ Ratio in the Liver

	Control	Ethanol
WT	342.9 ± 26.9	178.4 ± 42.3[a]
MT-TG	334.4 ± 50.8	154.6 ± 30.5[a]

Note. Three doses of ethanol were administrated by gavage at 5 g/kg, and hepatic $NAD^+/NADH$ ratio was estimated by enzymatically measuring lactate and pyruvate concentrations. Results are means ± SD (n = 5–7). WT, wild-type mice; MT-TG, MT transgenic mice.
[a] Significantly different from controls ($P < 0.05$).

Table II. Effect of Acute Ethanol Treatment on Microsomal CYP2E1 Activity in the Liver

	4-nitrocatechol (nmol/min/mg)	
	Control	Ethanol
WT	0.73 ± 0.10	1.85 ± 0.23[a]
MT-TG	0.75 ± 0.15	2.04 ± 0.33[a]

Note. Three doses of ethanol were administered by gavage at 5 g/kg, and CYP2E1 activities were estimated by colorimetrically measuring hydroxylation of p-nitrophenol to 4-nitrocatechol, a reaction catalyzed specifically by CYP2E1. Results are means ± SD (n = 5–7). WT, wild-type mice; MT-TG, MT transgenic mice.
[a] Significantly different from controls ($P < 0.05$).

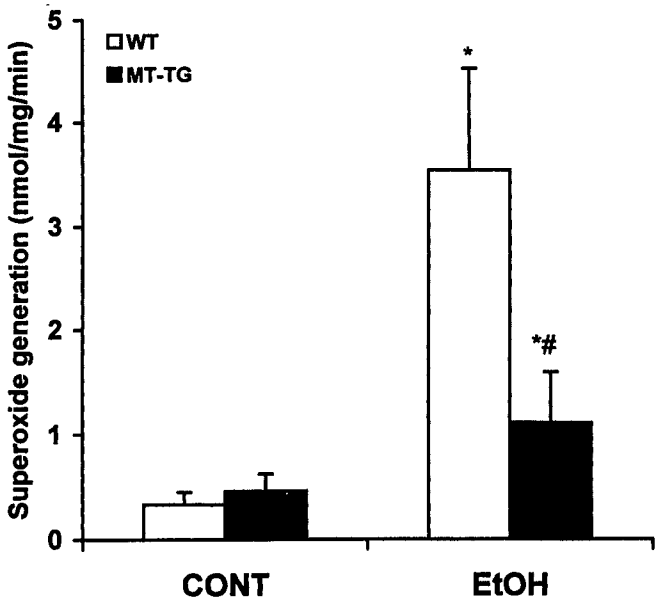

Figure 8. Hepatic superoxide content after acute ethanol administration. Three doses of ethanol were administrated by gavage at 5 g/kg, and accumulation of superoxide anion in the liver was quantified using a cytochrome reduction assay. Results are means ± SD (n = 5–7). *, Significantly different between control and ethanol treatment ($P < 0.05$). #, Significantly different between MT-TG and wild-type mice ($P < 0.05$).

this reactive oxygen species (13). Thus, it is possible that overproduction of superoxide anion due to NADH and CYP2E1 is responsible for the observed oxidative injury induced by ethanol in the liver. MT would scavenge superoxide, thereby inhibiting the chain reaction that leads to generation of other reactive oxygen species. The antioxidant action of MT would be highly responsible for the hepatic protection against ethanol toxicity. The data obtained indeed elucidate such a scenario. However, to demonstrate that MT directly scavenges superoxide *in vivo* is rather difficult, if not impossible at present. Further studies are required to address this important issue.

Supplementation with antioxidants as protection against ethanol-induced liver injury has been widely investigated (40). Although most studies have suggested that supplementing antioxidants could suppress ethanol-induced liver injury, there is no antioxidant that has been advised for clinical treatment. Nevertheless, developing antioxidant therapy is an important strategy to improve the prevention and treatment of alcoholic liver injury. Based on the results of our study and previous observations, we speculate that MT may be useful in the prevention and therapy of alcoholic liver injury. In particular, MT is highly inducible by a number of physiological and pharmacological agents. Therefore, the basis for developing pharmaceutical methods to increase hepatic MT concentrations already exists. Exploring the potential of MT in protecting against alcoholic liver injury would likely result in a novel approach to this clinical problem.

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