

A dual effect of *N*-acetylcysteine on acute ethanol-induced liver damage in mice

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Abstract

Reactive oxygen species (ROS) have been associated with acute ethanol-induced liver damage. *N*-acetylcysteine (NAC) is a glutathione (GSH) precursor and direct antioxidant. In this study, we investigated the effects of NAC on acute ethanol-induced liver damage. Female ICR mice were administered by gavage with a single dose of ethanol (6 g/kg). NAC was administered in two different modes. In mode A, mice were injected with different doses of NAC at 30 min before ethanol. In mode B, mice were injected with different doses of NAC at 4 h after ethanol. Acute ethanol-induced liver damage was estimated by measuring serum alanine aminotransferase (ALT) activity and histopathological changes. Result showed that a single dose of ethanol (6 g/kg) caused a significant increase in serum ALT activity, followed by microvesicular steatosis and necrosis in mouse liver. Pretreatment with NAC significantly protected against acute ethanol-induced liver damage in a dose-independent manner. Correspondingly, pretreatment with NAC significantly attenuated acute ethanol-induced lipid peroxidation and GSH depletion and inhibited hepatic TNF- α mRNA expression. By contrast, post-treatment with NAC aggravated ethanol-induced hepatic lipid peroxidation and worsened acute ethanol-induced liver damage in a dose-dependent manner. Taken together, NAC has a dual effect on acute ethanol-induced liver damage. Pretreatment with NAC prevent from acute ethanol-induced liver damage via counteracting ethanol-induced oxidative stress. When administered after ethanol, NAC might behave as a pro-oxidant and aggravate acute ethanol-induced liver damage.

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1. Introduction

Acute alcoholic hepatitis is a serious disease, with a mortality of up to 60% in the first 4 weeks of diagnosis in severe cases [1]. Lack of understanding of mechanism of alcoholic liver injury has hampered the development of effective treatments. Recently, the progress on the mechanism involved in the pathogenesis of alcoholic liver injury has demonstrated that alcoholic liver injury is a result of complex pathophysiological events involving various types of cells, such as neutrophils, endothelial cells, Kupffer cells, and hepatocytes, and different injurious factors such as endotoxin (lipopolysaccharide, LPS), oxidative stress, cytokines, and proteases [2–4].

Ethanol deleteriously affects the anatomical and functional integrity of intestinal mucosa and increases intestinal permeability, thus allowing gut-derived endotoxin LPS to escape into the blood [5–9]. Numerous studies demonstrated that LPS stimulates Kupffer cells to generate reactive oxygen species (ROS), such as superoxide (O_2^-) and nitric oxide (NO) [10,11]. Elevated levels of O_2^- and NO in Kupffer cells are responsible for activation of NF- κ B, releases of TNF- α , and, eventually, liver injury [12–16]. On the other hand, ethanol is a potent inducer of P450 2E1. Several studies

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demonstrated that P450 2E1-dependent formation of free radicals is involved in ethanol-induced liver damage [17,18]. ROS might play an important role on ethanol-induced liver damage [19–22].

N-acetylcysteine (NAC) is a glutathione (GSH) precursor and direct antioxidant. As a potent antioxidant, NAC directly scavenges hydrogen peroxide (H₂O₂), hydroxyl free radicals (•OH) and hypochloric acid (HOCl) in vitro [23]. NAC also decreases free radical levels by increasing GSH synthesis [24,25]. Several studies showed that NAC inhibited LPS-induced inducible nitric oxide synthase (iNOS), TNF- α expression and NF- κ B activity [26,27]. Our earlier study showed that pretreatment with NAC attenuated LPS-induced downregulation of *pregnane \times receptor* and *cytochrome P450 3a11* gene expressions in adult mouse liver, placenta and fetal liver [28–30]. Clinically, NAC has been successfully used in adult respiratory distress syndrome [31].

In the present study, we investigated the effects of NAC on acute ethanol-induced liver damage in mice. Our results found that pretreatment with NAC protected against acute ethanol-induced liver damage via counteracting ethanol-induced oxidative stress and inhibiting TNF- α expression in mouse liver, whereas post-treatment with NAC aggravated acute ethanol-induced liver damage.

2. Materials and methods

2.1. Chemicals

Ethanol, *N*-acetylcysteine (NAC) and DL-buthionine-(SR)-sulfoximine (BSO) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained from Sigma if not otherwise stated.

2.2. Animals and treatments

The ICR mice (6–8 weeks-old; male mice: 22–24 g; female mice: 20–22 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 \pm 5%) environment for a period of 1 week before use.

On the day prior to experiments, mice were fasted overnight. In the initial experiments, mice were administered by gavage with a single dose of ethanol (6 g/kg) and sacrificed at 3, 6, 9, and 12 h after ethanol. In subsequent experiments, mice administered with ethanol (6 g/kg) received different doses of NAC. NAC was administered in two different modes. In mode A, mice were injected with different doses of NAC (75, 150 or 300 mg/kg, i.p.) at 30 min before ethanol. In mode B, mice were injected with different doses of NAC (75, 150 or 300 mg/kg, i.p.) at 4 h after ethanol. The control mice received saline only. The mice were sacrificed either at 1.5 or 6 or 12 h

after ethanol. Blood serum was collected for measurement of alanine aminotransferase (ALT). Livers were dissected for total RNA extraction, GSH and TBARS measurement and histopathological examination.

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Alanine aminotransferase assay

Plasma alanine aminotransferase (ALT) activity was colorimetrically measured using a Diagnostic kit (Sigma Chemical Company, St. Louis, MO) according to the instruction provided.

2.4. Histopathological examination

Liver tissues were fixed with 10% neutral formalin and embedded in paraplast. Tissue sections of 5 μ m were cut and stained by hematoxylin and eosin.

2.5. Lipid peroxidation assay

Lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously [32]. Liver tissue was homogenized in 9 volumes of 50 mmol/l Tris-HCl buffer (pH 7.4) containing 180 mmol/l KCl, 10 mmol/l 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 h. 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 \times g for 10 min, absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,5,5-tetraethoxypropane as standard.

2.6. Determination of glutathione content

The glutathione (GSH) was determined by the method of Griffith [33]. Proteins of 0.4 ml liver homogenates were precipitated by the addition of 0.4 ml of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm at 4 °C for 5 min. A 400 μ l of the supernatant was combined with 0.4 ml of 300 mM Na₂HPO₄, and the absorbance at 412 nm was read against a blank consisting of 0.4 ml supernatant plus 0.4 ml H₂O. Then, 100 μ l DTNB (0.02%, w/v; 20 mg DTNB in 100 ml of 1% sodium citrate) was added to the blank and sample. Absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic

sample. GSH values were expressed as nmol mg⁻¹ protein. Protein content was measured according to the method of Lowry et al. [34].

2.7. Isolation of total RNA and RT

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNase-free DNase (Promega) was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. Total RNA was stored at -80 °C. For the synthesis of cDNA, 2.0 µg of total RNA from each sample was resuspended in a 20-µl final volume of reaction buffer, which contained 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM MgCl₂, 10 mM of each dNTP and 0.5 mg oligo(dT)₁₅ primer (Promega). After the reaction mixture reached 38 °C, 400 units of RT (Promega) was added to each tube and the sample was incubated for 60 min at 38 °C. Reverse transcription was stopped by denaturing the enzyme at 95 °C.

2.8. PCR amplification

The final PCR mixture contained 2.5 µl of cDNA, 1 × PCR buffer, 1.5 mM MgCl₂, 200 µM dNTP mixture, 1 U of Taq DNA polymerase, 1 µM sense and antisense primers, and sterile water to 50 µl. The reaction mixture was covered with mineral oil. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as an internal positive-control standard. Following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described [28]. GAPDH, 5'-GAG GGG CCA TCC ACA GTC TTC-3' and 5'-CAT CAC CAT CTT CCA GGA GCG-3'; TNF-α, 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' and 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'. The sizes of amplified PCR products were 340 bp for GAPDH and 307 bp for TNF-α. Number of cycles and annealing temperature were optimized for each primer pair. For GAPDH, amplification was initiated by 3 min of denaturation at 94 °C for one cycle, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. For TNF-α, amplification was initiated by 3 min of denaturation at 94 °C for one cycle, followed by 30 cycles each of denaturation at 94 °C for 45 s, annealing of primer and fragment at 60 °C for 45 s, and primer extension at 72 °C for 1 min. A final extension of 72 °C for 10 min was included. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, St. Louis, MO) for 45 min. The pBR322 DNA digested with *AluI* was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma, St. Louis, MO) TBE buffer.

2.9. Statistical analysis

Quantified data were expressed as means ± S.E.M. at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences between the treated animals and the control and statistical significance.

3. Results

3.1. Effects of NAC on acute ethanol-induced liver damage

Acute ethanol-induced liver damage was evaluated by measuring serum ALT activity and histopathological changes. As shown in Fig. 1, a single dose of ethanol administration significantly increased serum ALT activity in a time-dependent manner. Microvesicular steatosis and necrosis were observed in mouse liver at 6 h after ethanol treatment (Fig. 2). When administered as a pretreatment, NAC protected against ethanol-induced increase in serum ALT activity in a dose-independent manner (Fig. 3A), which was not blocked by pretreatment with BSO (Fig. 4). Ethanol-induced microvesicular steatosis and necrosis also were attenuated by pretreatment with NAC. By contrast, post-treatment with NAC significantly aggravated ethanol-induced increase in serum ALT activity (Fig. 3B) and histopathological changes (Fig. 2).

3.2. Effects of NAC on ethanol-induced lipid peroxidation

Lipid peroxidation was quantified by measuring TBARS. As shown in Fig. 5, ethanol administration significantly

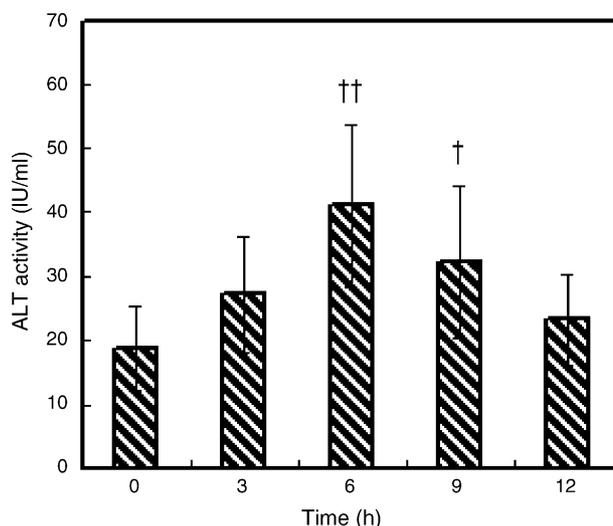


Fig. 1. The effects of ethanol on serum ALT activity. Mice were administered by gavage with ethanol (6 g/kg) and sacrificed at 3, 6, 9, and 12 h after ethanol. Serum ALT activity was measured as described in Section 2. Data were expressed as means ± S.E.M. ($n = 12$). † $P < 0.05$, †† $P < 0.01$ as compared with control group.

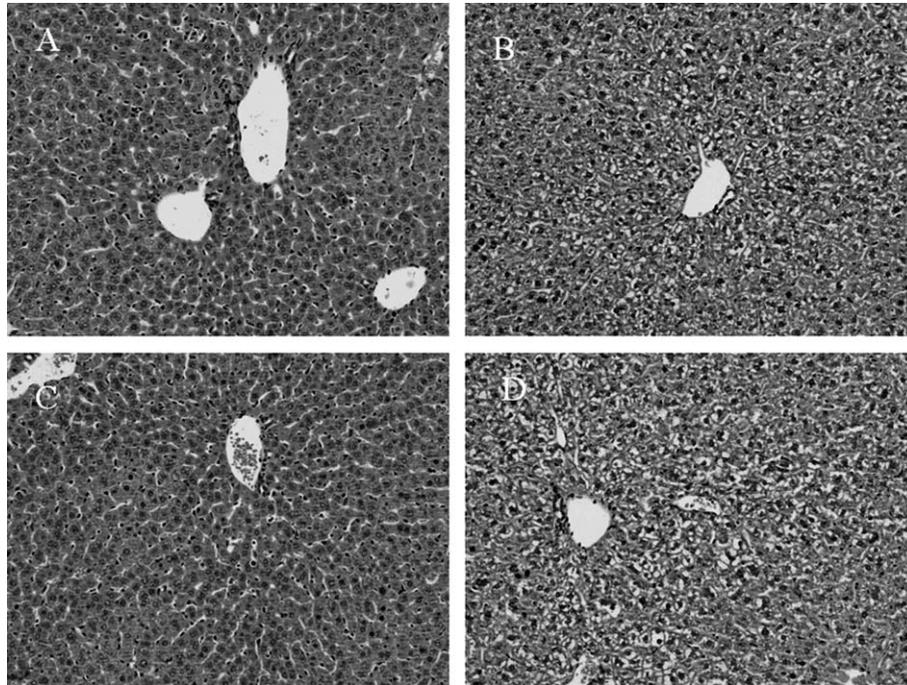


Fig. 2. The effects of NAC on ethanol-induced histopathological changes in mouse liver. Mice administered by gavage with ethanol (6 g/kg) received a single dose of NAC (150 mg/kg, i.p.) either 30 min prior to ethanol or 4 h after ethanol. Mice were sacrificed at 6 h after ethanol. (A) Control; (B) alcohol; (C) pretreatment with NAC; (D) post-treatment with NAC. Hematoxylin and eosin staining; magnification, 200 \times .

increased hepatic TBARS content as early as 3 h after ethanol treatment. A time-dependent increase in TBARS content was observed up to 12 h. When administered at 30 min before ethanol, NAC significantly attenuated ethanol-induced increase in hepatic TBARS level in a dose-independent manner (Fig. 6A), whereas post-treatment with ethanol aggravated ethanol-induced lipid peroxidation in mouse liver (Fig. 6B).

3.3. Effects of NAC on ethanol-induced GSH depletion

The effects of ethanol on hepatic GSH content are presented in Fig. 7. Results showed that acute ethanol exposure significantly decreased hepatic GSH content as early as 3 h after ethanol administration. Pretreatment with NAC significantly attenuated ethanol-induced GSH depletion (Fig. 8A). However, post-treatment with NAC had no effect on ethanol-induced hepatic GSH depletion (Fig. 8B).

3.4. Effects of NAC on ethanol-induced hepatic TNF- α expression

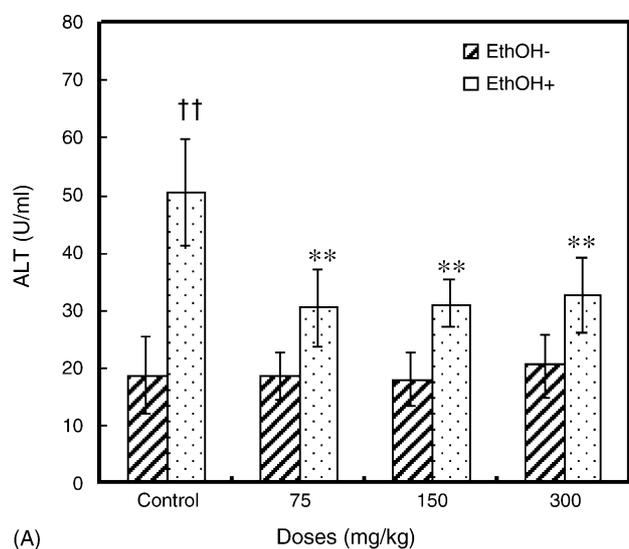
The effects of NAC on ethanol-induced hepatic TNF- α expression are presented in Fig. 9. Ethanol markedly increased hepatic TNF- α mRNA level. When administered at 30 min before ethanol, NAC significantly attenuated ethanol-induced hepatic TNF- α mRNA expression. However, when administered after ethanol, NAC had little effect on ethanol-induced TNF- α mRNA expression in mouse liver.

4. Discussion

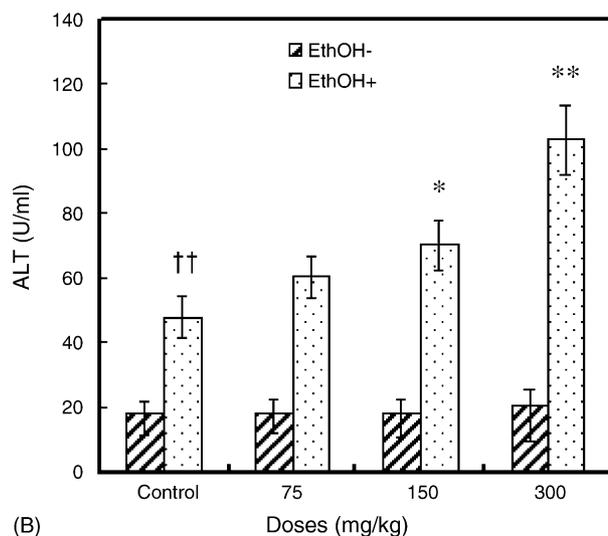
The present study investigated the effects of NAC on acute ethanol-induced liver damage. Results showed that pretreatment with NAC significantly attenuated ethanol-induced increase in serum ALT activity and histopathological changes. Furthermore, pretreatment with NAC significantly attenuated ethanol-induced increase in hepatic TBARS concentration, suggesting that NAC-mediated protection against acute ethanol-induced liver damage is, at least in part, associated with decreased lipid peroxidation in mouse liver.

The antioxidant activity of NAC primarily involves two mechanisms [23]: (1) NAC acts as a free radical scavenger, directly scavenges hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and hydroxyl radical ($\bullet\text{OH}$), and (2) NAC acts as a precursor of GSH to facilitate intracellular GSH synthesis. In the present study, we analyzed the effects of acute ethanol exposure on GSH content in mouse liver. As expected, a single dose (6.0 g/kg) of ethanol markedly decreased GSH level in mouse liver. Furthermore, pretreatment with NAC significantly attenuated ethanol-induced hepatic GSH depletion. However, the protective effect of NAC on acute ethanol-induced liver damage was not blocked by DL-buthionine-(SR)-sulfoximine (BSO), an inhibitor of GSH synthesis, suggesting that NAC-mediated protection against acute ethanol-induced liver damage is most likely due to its potent ROS-scavenging effect but not attributed to increased GSH synthesis.

Numerous studies demonstrated that TNF- α plays an important role in acute ethanol-induced liver damage [35].



(A)



(B)

Fig. 3. The effects of NAC on serum ALT activity. (A) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) 30 min prior to ethanol. Mice were sacrificed at 6 h after ethanol. (B) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) at 4 h after ethanol. Mice were sacrificed at 6 h after ethanol. Serum ALT activity was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n=12$). †† $P<0.01$ as compared with control group. * $P<0.05$, ** $P<0.01$ as compared with ethanol-treated group.

Animal experiment showed that acute ethanol administration significantly increased serum TNF- α levels. Acute ethanol-induced liver damage was preceded by rapidly increases in serum TNF- α levels [6]. NAC is an inhibitor of TNF- α production [24,36,37]. The present study investigated the effects of acute ethanol administration on hepatic TNF- α mRNA expression. Results showed that a single dose of ethanol dramatically increased TNF- α mRNA level in mouse liver. Pre-treatment with NAC significantly attenuated ethanol-induced increase in hepatic TNF- α mRNA level, suggesting that NAC-mediated protection against acute ethanol-induced liver

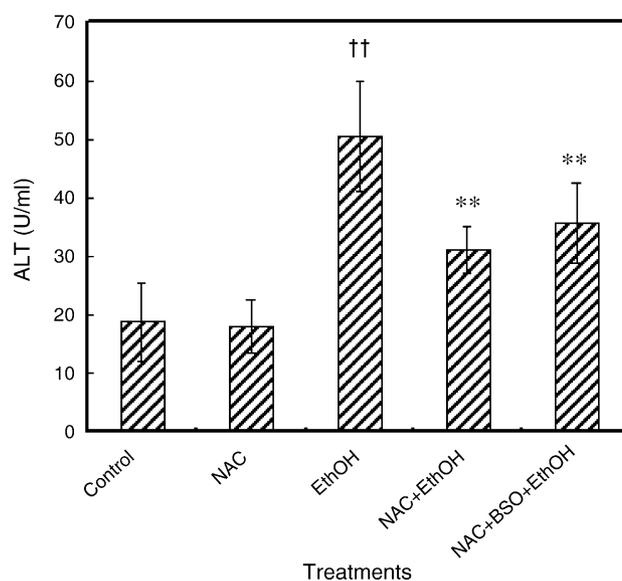


Fig. 4. The effects of BSO on NAC-mediated protection against acute ethanol-induced liver damage. Mice administered by gavage with ethanol (6 g/kg) received a single dose of NAC (150 mg/kg, i.p.) 30 min prior to ethanol. Mice were sacrificed at 6 h after ethanol. In BSO+NAC group, mice were injected with two doses of BSO, one (100 mg/kg, i.p.) at 12 h before NAC and the other (100 mg/kg, i.p.) at 1 h before NAC. Mice were sacrificed at 6 h after ethanol. Serum ALT activity was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n=12$). †† $P<0.01$ as compared with control group. * $P<0.05$, ** $P<0.01$ as compared with ethanol-treated group.

damage might also be associated with inhibition of TNF- α production in mouse liver.

The present study found that post-treatment with NAC had no effect on ethanol-induced GSH depletion and TNF- α

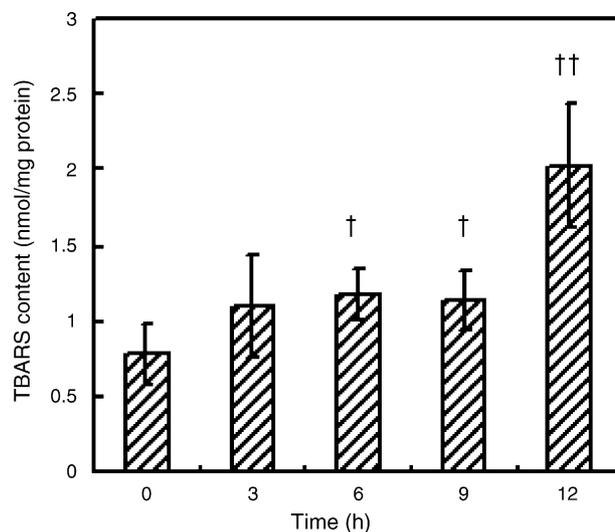
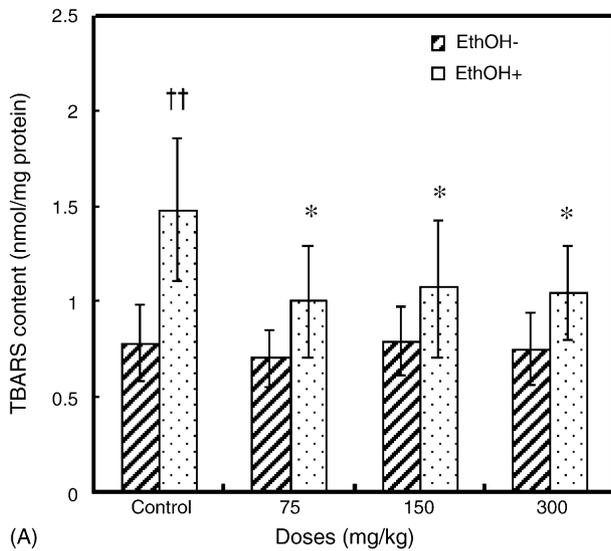
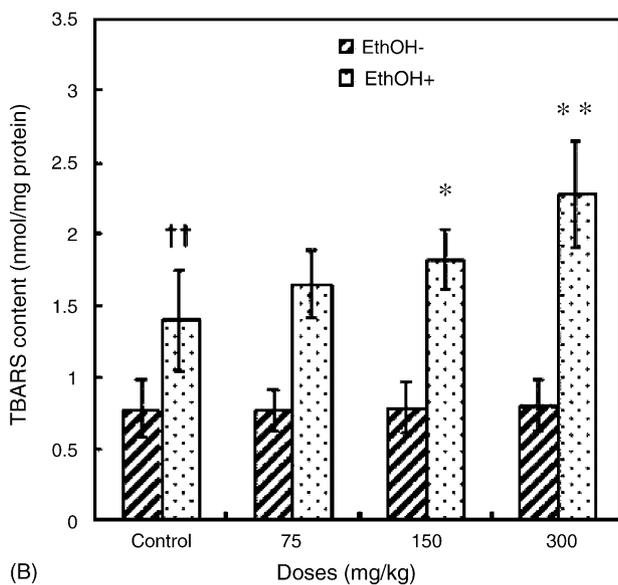


Fig. 5. The effects of ethanol on hepatic TBARS content. Mice were administered by gavage with ethanol (6 g/kg) and sacrificed at 3, 6, 9, and 12 h after ethanol. Hepatic TBARS content was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n=12$). † $P<0.05$, †† $P<0.01$ as compared with control group.



(A)



(B)

Fig. 6. The effects of NAC on hepatic TBARS content. (A) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) 30 min prior to ethanol. Mice were sacrificed at 12 h after ethanol. (B) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) at 4 h after ethanol. Mice were sacrificed at 12 h after ethanol. Hepatic TBARS content was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n = 12$). †† $P < 0.01$ as compared with control group. * $P < 0.05$, ** $P < 0.01$ as compared with ethanol-treated group.

mRNA expression. When administered at 4 h after ethanol, NAC aggravated ethanol-induced hepatic lipid peroxidation and worsened acute ethanol-induced liver damage.

Usually, NAC is mentioned as an “antioxidant”. However, NAC and other thiol chemicals have been demonstrated to be also pro-oxidants [38,39]. According to the report by Sprong et al. [40], NAC behaves either as anti- or pro-oxidants depending on the dose administered. Low dose (275 mg/kg in 24 h) of NAC protected rats against LPS-mediated oxida-

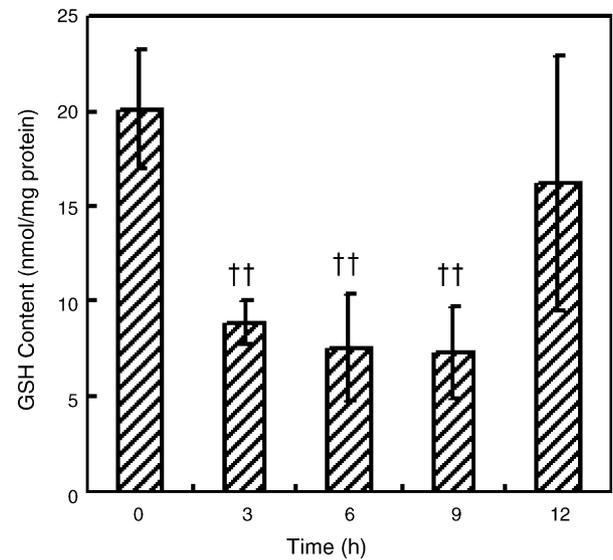


Fig. 7. The effects of ethanol on hepatic GSH content. Mice were administered by gavage with ethanol (6 g/kg) and sacrificed at 3, 6, 9, and 12 h after ethanol. Hepatic GSH content was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n = 12$). †† $P < 0.01$ as compared with control group.

tive stress, whereas high dose NAC (900 mg/kg in 24 h) increased LPS-induced lung injury and mortality. Chan et al. [41] found that NAC inhibited LPS-induced activation of the mitogen-activated protein kinases (MAPKs) in a serum-depleted environment (0.1% fetal bovine serum). By contrast, NAC enhanced LPS induction of p38mapk and JNK phosphorylation in the presence of 10% serum. We hypothesize that local redox environment might influence the effect of NAC. Before ethanol, the body is in intracellular redox balance. When administered before ethanol, NAC behaved as antioxidant and significantly attenuated ethanol-induced oxidative stress in mouse liver. However, when administered after ethanol, NAC may interact with ROS generated by ethanol and generate thiyl radicals, which, in turn, may impart a pro-oxidant function. Actually, the presence of metals, such as Cu(II), and the presence of ROS, such as H_2O_2 , potentiate “auto-oxidize” process of NAC [42]. Furthermore, as it has undergone auto-oxidation, thiol no longer acts as an “antioxidant”. Once initiated, these reactions can produce additional ROS including $O_2^{\cdot-}$, H_2O_2 , and $\cdot OH$. Thus, when administered at 4 h after ethanol, NAC behaved as pro-oxidant, aggravated ethanol-induced lipid peroxidation, and eventually worsened acute ethanol-induced liver damage. These results are in agreement with our recent report [43], in which we found that pretreatment with NAC prevented from LPS-induced intra-uterine fetal death and reverses LPS-induced fetal growth and skeletal development retardation, whereas post-treatment with NAC aggravates LPS-induced preterm labor.

In summary, the present study found that NAC has a dual effect on acute ethanol-induced liver damage. The effects of NAC depend on the schedule of NAC administration. Pre-

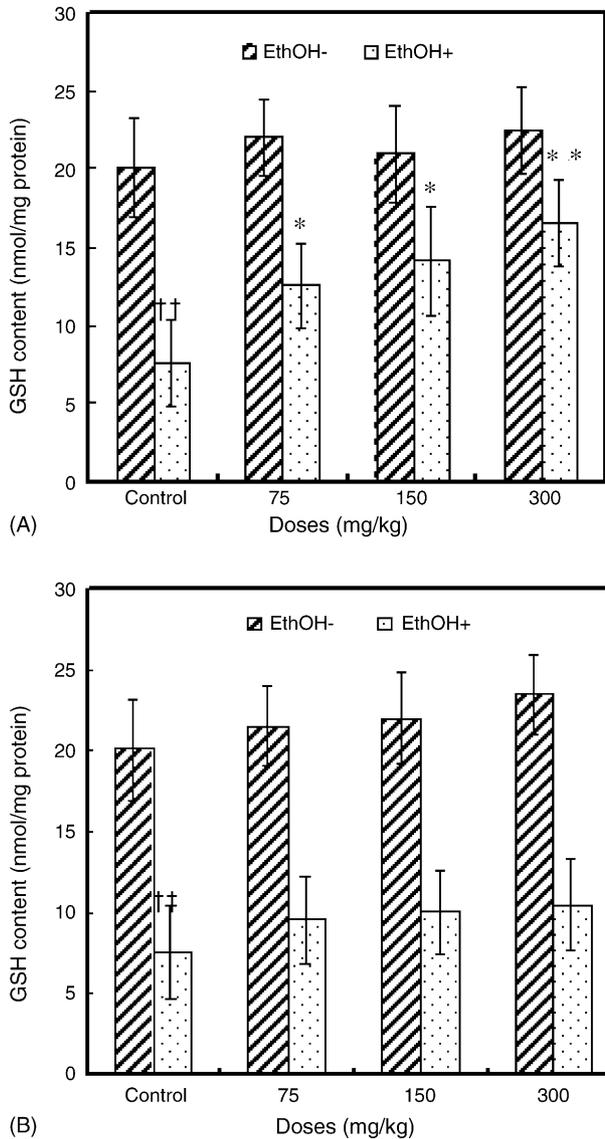


Fig. 8. The effects of NAC on hepatic GSH content. (A) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) 30 min prior to ethanol. Mice were sacrificed at 6 h after ethanol. (B) Mice administered by gavage with ethanol (6 g/kg) received different doses of NAC (75, 150, 300 mg/kg, i.p.) 4 h after ethanol. Mice were sacrificed at 6 h after ethanol. Hepatic GSH content was measured as described in Section 2. Data were expressed as means \pm S.E.M. ($n=12$). †† $P < 0.01$ as compared with control group. * $P < 0.05$ as compared with ethanol-treated group.

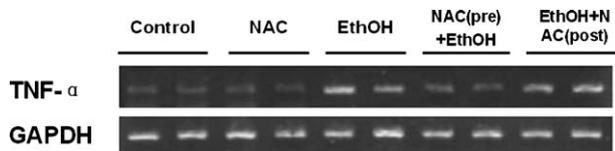


Fig. 9. The effects of NAC on ethanol-induced hepatic TNF- α expression. Mice administered by gavage with ethanol (6 g/kg) received NAC (150 mg/kg, i.p.) either at 30 min before or 30 min after ethanol. Mouse livers were excised at 1.5 h after ethanol treatment for total RNA extraction. TNF- α mRNA levels were determined using RT-PCR.

treatment with NAC protects against acute ethanol-induced liver damage via counteracting hepatic oxidative stress and inhibiting TNF- α expression in mouse liver. However, when administered after ethanol, NAC behaves as a pro-oxidant and worsens acute ethanol-induced liver damage.

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