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Protective effect of bicyclol on acute alcohol-induced liver injury in mice

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Abstract

Oxidative stress, cytokine over expression and Kupffer cell activation are well-documented pathological factors in the development of alcoholic liver disease. Bicyclol is a novel synthetic anti-hepatitis drug with anti-oxidative and anti-inflammatory property. The present study was to investigate the effect of bicyclol on acute alcohol-induced liver injury and related mechanisms in mice. Bicyclol (200, 300 mg/kg) was given to mice by gavage for three times. Alcohol (6 g/kg) was administered orally 1 h after the last dose of bicyclol. All animals were sacrificed at different time points after alcohol administration. Liver injury was evaluated by biochemical and histopathological examination. Lipid peroxidation and the activity of antioxidants were measured by spectrophotometric method. Expression of cytokines and CD14 were determined by enzyme-linked immunosorbent assay, reverse transcriptional-polymerase chain reaction and immunohistochemical staining. As a result, bicyclol pretreatment significantly protected against acute alcohol-induced liver injury as evidenced by the decrease of elevated serum alanine aminotransferase and hepatic triglyceride, and the attenuation of histopathological changes in mice. In addition, bicyclol remarkably alleviated the formation of thiobarbituric acid-reactive substance and restored impaired antioxidants, including glutathione, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Over-expression of cytokines, such as tumor necrosis factor α and interleukin 1β, elevated plasma endotoxin level, and up-regulation of CD14 were also suppressed by bicyclol in alcohol-intoxicated mice. The protective effect of bicyclol on alcohol-induced hepatotoxicity was mainly due to its ability to attenuate oxidative stress, suppress the cytokine expression at both protein and gene level, and inhibit the activation of Kupffer cells by decreasing plasma endotoxin level and CD14 expression.

Keywords: Bicyclol; Alcohol; Oxidative stress; Endotoxin; Cytokines

1. Introduction

Alcohol is one of the main causes of end-stage liver disease worldwide, and alcoholic liver disease is the second most common reason for liver transplantation in the United States (Mandayam et al., 2004). Due to increased frequency of drinking and change of diet construction, such as the increase of fat content, the incidence of alcoholic liver disease has increased in China, becoming another important risk factor for morbility and mortality in addition to viral hepatitis (Zhuang and Zhang, 2003). Except for the combination of abstinence from alcohol and supportive care (Bouneva et al., 2003), however, there is no satisfactory therapy for alcoholic liver disease at present.

The spectrum of alcoholic liver disease ranges from fatty liver to alcoholic hepatitis and ultimately to fibrosis and cirrhosis (Tuma and Sorrell, 2004). Much progress has been made in understanding the mechanisms of alcoholic liver disease, suggesting a complex pathological process that subsequent to alcohol metabolism in the liver and involves multiple types of cells, such as hepatocytes, Kupffer cells, neutrophils, endothelial cells and hepatic stellate cells (Tsukamoto and Lu, 2001).

There are three known metabolic enzyme systems, each located in different subcellular compartment, that participate in the oxidation of alcohol: alcohol dehydrogenase (ADH) in cytoplasm, microsomal ethanol oxidizing system (MEOS) in endoplasmic reticulum, and catalase in peroxisome (Lieber and Abittan, 1999; Lieber, 1997). All of them lead to over production of reactive oxygen species, including superoxide, peroxide, and

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hydroxyl radical, which can cause complete degradation of lipids, proteins, and DNA (Wu and Cederbaum, 2003). In addition to reactive oxygen species production, alcohol exposure also impair enzymatic and non-enzymatic mechanisms that protect cells against reactive oxygen species, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase (GR), and glutathione (GSH) (Wu and Cederbaum, 2003). Enhanced reactive oxygen species production and compromised antioxidant activity result in oxidative stress, which has been demonstrated to play an important role in alcohol-induced liver injury (Dey and Cederbaum, 2006; Cederbaum, 2001).

The significant role of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL)-1β has been indicated in both clinical and experimental alcoholic liver disease (McClain et al., 1999; Thurman et al., 1999; Tilg and Diehl, 2000). Kupffer cells, the resident macrophages in liver (Wheeler, 2003), are believed to be the main source of above cytokines in alcoholic liver disease. Endotoxin has been found to activate Kupffer cells by binding with CD14 (Wright et al., 1990), which distributes exclusively on the outside of Kupffer cells, and triggers a cascade of biochemical signals. Accumulative evidences have demonstrated that alcohol exposure increases endotoxin level in blood by both preventing its clearance from circulation and enhancing its absorption from intestine (Rivera et al., 2003; Nolan, 1975; Adachi et al., 1994).

Bicyclol (4, 4'-dimethoxy-5, 6, 5' 6'-bis (dimethylene-dioxy)-2-hydroxymethyl-2'-methoxy carbonyl biphenyl; Fig. 1) is a novel synthetic anti-hepatitis drug for the treatment of chronic hepatitis B patients in China (Liu, 2001). It has been reported that bicyclol has protective effect against experimental liver injury induced by various chemical toxins including CCl_4, D-galactosamine, concanavalin A and acetaminophen. The hepatoprotective mechanisms of bicyclol involve the clearance of reactive oxygen species, regulation of cytokine secretion, and inhibition of apoptosis induced by immunological injury, etc. (Liu, 2001).

The purpose of the present study was to determine whether bicyclol had protective effect on acute alcohol-induced liver injury, a rodent model represents early pathological changes in alcoholic liver disease, and investigate related mechanisms on oxidative stress, cytokine expression and Kupffer cell activation.

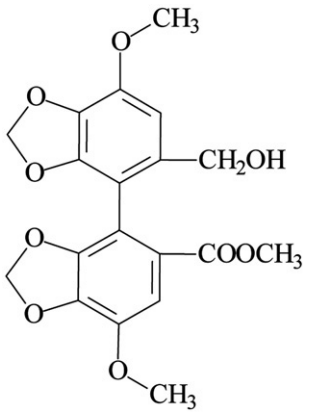

Fig. 1. Chemical structure of bicyclol.

2. Materials and methods

2.1. Reagents

Bicyclol was kindly provided by Beijing Union Pharmaceutical Plant (purity > 99%). Alcohol was purchased from Beijing Qixing Alcohol Co., Ltd. (China). Alanine aminotransferase (ALT) and triglyceride kits were obtained from BHKT Chemical Reagent Co., Ltd. (China). SOD, catalase, GR and GSH-peroxidase kits were obtained from Nanjing Jiancheng Bioengineering Institute (China). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (USA). Endotoxin assay kit was purchased form Xiamen Houshiji Ltd. (China). Rabbit anti-mouse TNF-α antibody and anti-mouse CD14 antibody were products of Boster Biological Technology Ltd. (China). Trizol was obtained from BioDev Tech Co., Ltd. (China). Reverse transcriptional-polymerase chain reaction (RT-PCR) kits were purchased from Takara Biotechnology Co., Ltd. (Japan). Other chemicals were of analytical grade and were obtained from the local market.

2.2. Treatment of animals

Male ICR mice weighing 22–24 g were obtained from Beijing Vital River Experimental Animal Co., Ltd. The mice were maintained at 22 °C with a 12-hour light/dark cycle and had free access to rodent chow and tap water. All experimental procedures were in compliance with the guidelines of China for animal care, which was conformed to the internationally accepted principles in the care and use of experimental animals.

In bicyclol treated group, mice were given bicyclol 200, 300 mg/kg (suspended in 5% carboxymethyl cellulose) by gavage for three times in two consecutive days, other groups received an equal volume of vehicle as control. All animals were administered orally with alcohol 6 g/kg 1 h after the last dose of bicyclol except mice in normal control group. The animals were sacrificed at different time points that indicated in the following contents. Blood samples were collected for the measurement of serum ALT and plasma endotoxin level. Two pieces of tissues from the same lobe of liver in each animal were fixed properly for histopathological and immunohistochemical examinations respectively. The rest of liver tissues were stored at −80°C for biochemical assays, ELISA and RNA isolation.

2.3. Serum alanine aminotransferase and hepatic triglyceride assay

Serum ALT level was measured colorimetrically by a diagnostic kit according to the instructions provided.

Liver tissues were homogenized in 9 volumes of 0.9% NaCl and hepatic triglyceride content was determined by a commercial kit.

2.4. Histopathological examination

Liver tissues were fixed with 10% neutral formalin and embedded in paraplast. Tissue sections (5 μm) were cut and stained by hematoxylin and eosin.

2.5. Lipid peroxidation assay

Lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance as previously described (Ohkawa et al., 1979). The concentration of thiobarbituric acid-reactive substance was calculated using 1, 1, 5, 5-tetraethoxypropane as standard and expressed as nmol/mg protein.

2.6. Hepatic GSH assay

Hepatic GSH content was determined as described by Ellman (Ellman, 1959). Liver homogenates (500 μl) were precipitated by adding 0.5 ml of 4% sulfosalicylic acid and centrifugation at 2858 ×g for 10 min. The supernatant (900 μl) was mixed with 0.004% DTNB (100 μl). After being vortexed and standing for 10 min at room temperature, absorbance of the mixture was read at 412 nm and GSH content was determined using a standard curve.

2.7. Preparation of hepatic subcellular fractions

Fresh liver tissues were washed with 0.9% NaCl and homogenized in 3 volumes (w/v) of 10 mM Tris–Acetate (pH 7.4) containing 0.25 m sucrose and 0.5 mM EDTA at 4 °C. After crude homogenate was centrifuged at 800 ×g for 10 min at 4 °C to remove the nuclear fraction and cell debris, the supernatant was centrifuged at 6500 ×g for 20 min at 4 °C. The mitochondrial fraction remained in the precipitate. The supernatant was subjected to a further centrifugation at 10,000 ×g for 60 min at 4 °C. The microsomal and cytosolic fractions were kept in the precipitate and supernatant respectively. The above hepatic subcellular fractions were stored in aliquants at −80 °C until the enzyme assay was conducted.

2.8. Determination of antioxidant enzyme activities

Cytosolic SOD, catalase, GR and mitochondrial GSH-peroxidase were measured by spectrophotometric analysis using commercial kits.

2.9. Liver TNF-α and IL-1β quantification

Liver samples for cytokine quantification were prepared as previously described (Wolf et al., 2001; Zhou et al., 2003). Briefly, liver samples were disintegrated in 4 volumes of ice-cold Ripa buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4]), containing protease inhibitors (1 μg/mL aprotinin, 1 0μg/mL leupeptin and 1 μg/mL pepstaitn), Dnase (0.05 mg/mL), and detergents (0.3% Triton X-100, 0.03% sodium dodecyl sulfate, 0.3% sodium deoxycholate). After incubation on ice for 30 min, samples were centrifuged twice at 20,000 ×g for 15 min at 4 °C. The resulting supernatants were harvested and stored at −80 °C until quantification of intrahepatic cytokines by murine ELISA kits. Liver lysates were adjusted to equal protein concentrations after quantified by the Coomassie blue method. The results were expressed as pg/mg protein.

2.10. Isolation of total liver RNA and RT-PCR for mRNA expression of TNF-α and IL-1β

Total RNA from liver tissue was isolated using Trizol reagent according to the manufacturer's instructions. A 500 ng of total RNA was used for cDNA synthesis and 10 μl of each reverse transcription product was added to 40 μl of reaction mixture containing 10 μl of 5×PCR buffer, 0.25 μl of 5 U/μl *Ex Taq*®DNA polymerase, 1 μl of 100 μM corresponding primers,

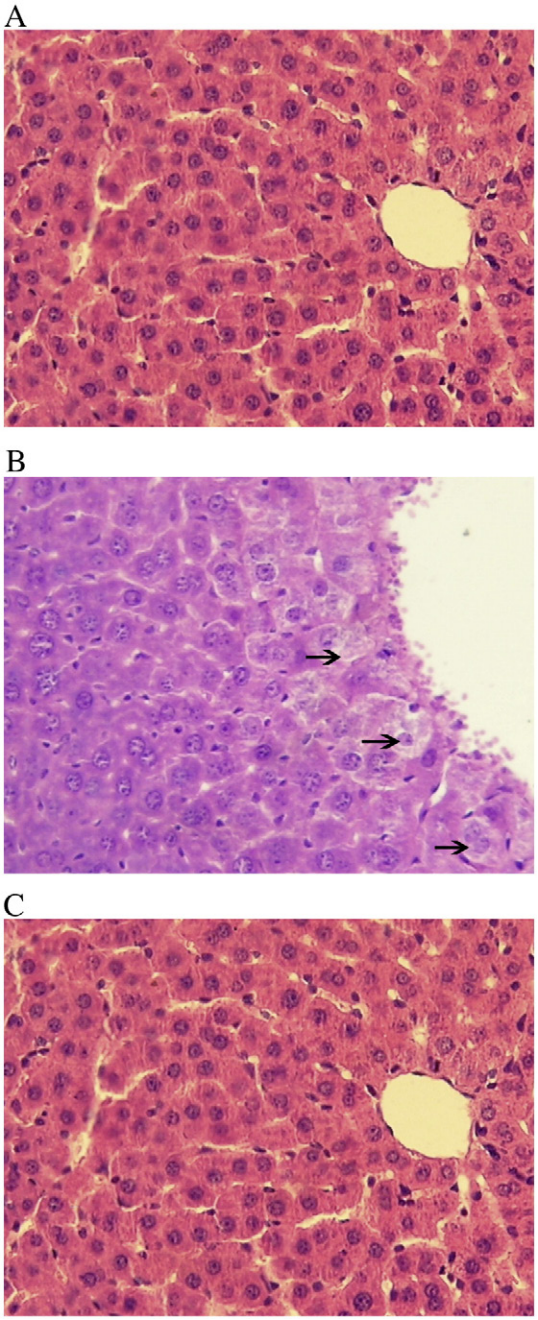

Fig. 2. Effect of bicyclol on liver injury induced by alcohol in mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 12 h after alcohol administration. (A) Control; (B) Alcohol; (C) Bicyclol. Alcohol treatment induced swelling and hydropic degeneration of hepatocytes (arrows). Hematoxylin and eosin staining; Original magnification, ×100.

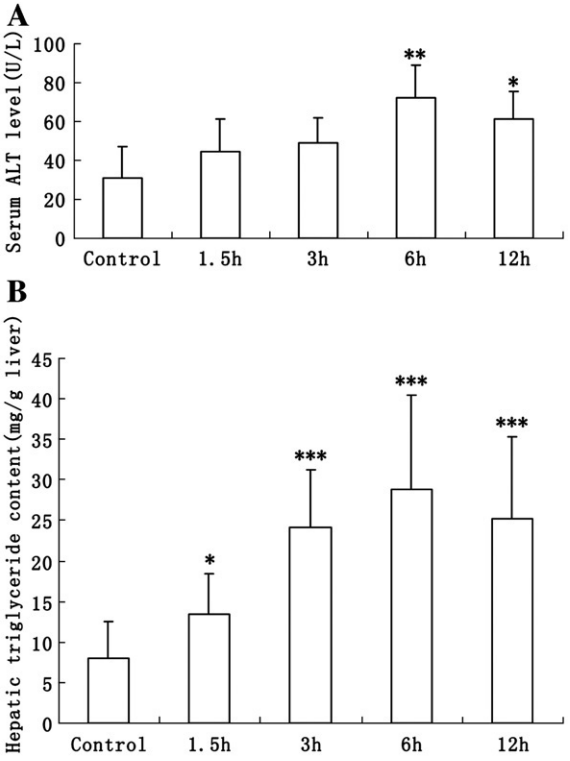

Fig. 3. Time-course changes of serum ALT and hepatic triglyceride in acute alcohol-intoxicated mice. Alcohol (6 g/kg) was administered to mice by gavage. The animals were sacrificed at 1.5, 3, 6, and 12 h after alcohol administration. Data were expressed as means±SD ($n=8$). *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ vs. control group.

and 27.75 μl of ddH_2O for PCR amplification. The following primers were synthesized by SBS Genetech: GAPDH, 5'-GTC TTC ACC ACC ATG GAG AAG GC-3' and 5'-ATT CAT TGT CAT ACC AGG AAA-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'; IL-1β, 5'-AGC CCA TCC TCT GTG ACT CAT G-3' and 5'-GCT GAT GTA CCA GTT GGG GAA C-3'; The size of amplified PCR products were 652 bp for GAPDH, 307 bp for TNF-α, and 420 bp for IL-1β. PCR was initiated at 94 °C for 2 min followed by 23–40 cycles at 94 °C for 30 s, 50–60 °C for 30 s, and 72 °C for 1 min. Number of cycles and annealing temperature for each primer pair were optimized. Number of cycles for GAPDH, TNF-α, and IL-1β was 23, 40, and 35 respectively, while the annealing temperature was 55 °C, 60 °C, and 56 °C. A final extension of 72 °C for 10 min was included. The amplified PCR products were subjected to electrophoresis at 100 V through 1.5% agarose gels for 40 min. The 100 bp DNA ladder was used as molecular marker. The bands were visualized with ethidium bromide and analyzed by BandScan.

2.11. Determination of plasma endotoxin level

Plasma endotoxin level was measured by a quantitative chromogenic end-point tachypleus amebocyte lysate endotoxin detection kit following the manufacturer's instructions. Briefly, plasma samples were diluted to1:10 with water/Tris–HCl buffer and heated at 70 °C for 10 min to denature endogenous endotoxin inhibitors. After centrifuged at 1270 ×g for 10 min, the supernatant was removed and incubated with limulus amebocyte lysate at 37 °C for 10 min, followed by incubation with the provided chromogenic substance for 6 min. The absorbance at 545 nm was measured after adding appropriate azo-reagents.

2.12. Immunohistochemical localization of liver TNF-α and CD14

Formalin-fixed, paraffin-embedded sections (10 μM) were mounted on glass slides. Sections were deparaffinized, incubated in 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 min, the sections were stained with polyclonal rabbit anti-TNF-α and anti-CD14 antibody at 4 °C overnight respectively, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody at 37 °C for 30 min. The antibody biding sites were visualized by incubation with DAB-H_2O_2 at room temperature for 10 min.

2.13. Statistical analysis

All data were expressed as mean±SD. Statistical analysis was performed using ANOVA and the Student–Newmann–Keuls post hoc test. Differences between groups were considered significant at $P<0.05$.

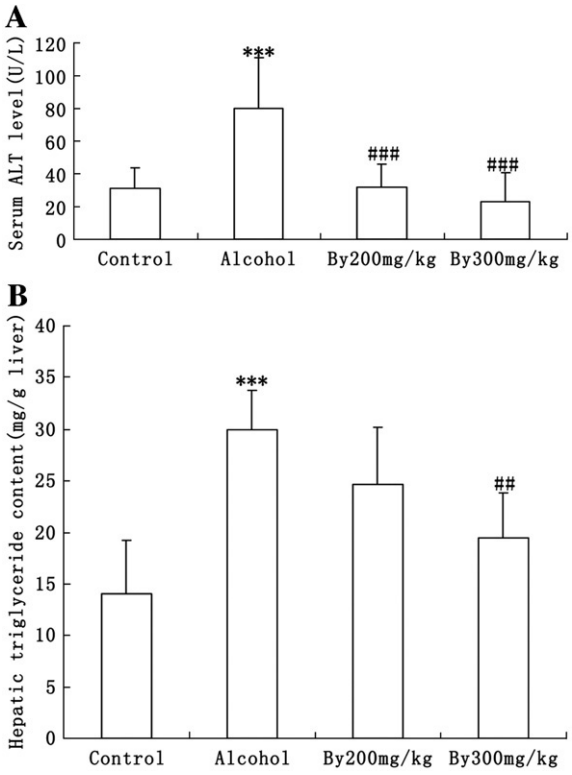

Fig. 4. Effect of bicyclol on the elevation of serum ALT and hepatic triglyceride levels in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 6 h after alcohol administration. Data were expressed as means±SD ($n=8$). ***, $P<0.001$ vs. control group; ##, $P<0.01$, ###, $P<0.001$ vs. alcohol group.

3. Results

3.1. Effect of bicyclol on acute liver injury induced by alcohol

Acute alcohol-induced liver injury was indicated by elevated serum ALT, hepatic triglyceride and liver pathological changes characterized by swelling and hydropic degeneration of hepatocytes around the central and interlobular veins. Pretreatment of the mice with bicyclol remarkably attenuated the above histopathological changes (Fig. 2). Serum ALT and hepatic triglyceride were increased by 2.3 and 3.5 fold respectively over those in normal mice at 6 h after administration of alcohol (Fig. 3). Administration with bicyclol significantly reduced the elevation of serum ALT and accumulation of hepatic triglyceride in a dose-dependent manner (Fig. 4).

3.2. Effect of bicyclol on hepatic lipid peroxidation induced by alcohol

Liver lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substance content. Hepatic thiobarbituric acid-reactive substance content was elevated from 6 h after alcohol administration and reached 2.7 fold of the control at 12 h. (Fig. 5). Pretreatment with bicyclol significantly attenuated alcohol-induced increasing of hepatic thiobarbituric acid-reactive substance by 32% (Fig. 6).

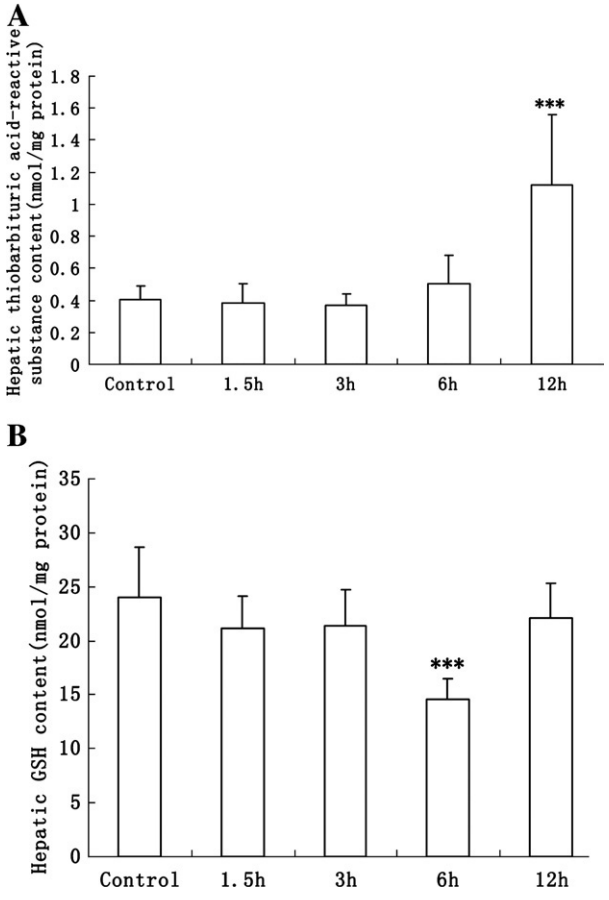

Fig. 5. Time-course changes of hepatic lipid peroxidation and GSH content in acute alcohol-intoxicated mice. Alcohol (6 g/kg) was administered to mice by gavage. The animals were sacrificed at 1.5, 3, 6, and 12 h after alcohol administration. Data were expressed as means±SD (n=8). ***, P<0.001 vs. control group.

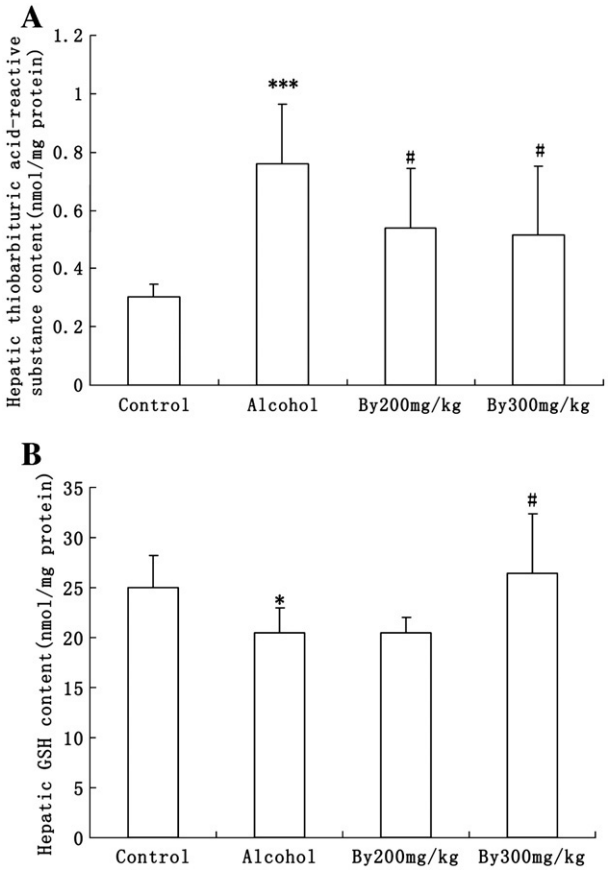

Fig. 6. Effect of bicyclol on hepatic lipid peroxidation and GSH depletion in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 12 h and 6 h after alcohol administration for thiobarbituric acid-reactive substance and GSH content determination respectively. Data were expressed as means±SD (n=8). *, P<0.05, ***, P<0.001 vs. control group; #, P<0.05 vs. alcohol group.

3.3. Effect of bicyclol on hepatic GSH depletion induced by alcohol

As shown in Fig. 5, hepatic GSH content was decreased as early as 1.5 h after alcohol treatment and only 40% of control was found at 6 h. Administration of bicyclol (300 mg/kg) protected against alcohol-induced hepatic GSH depletion, as evidenced by reversing to normal level (Fig. 6).

3.4. Effect of bicyclol on antioxidant enzyme activities in alcohol-intoxicated mice

A notable change of liver SOD, catalase, GR and GSH-peroxidase was observed as indicated by the decreasing of enzyme activity (35%, 18%, 49% and 45%) at 1.5 h after alcohol administration. Pretreatment with bicyclol significantly inhibited the decrease of SOD and GSH-peroxidase activity in a dose-dependent manner. In addition, high dose of bicyclol (300 mg/kg) showed protective effect against alcohol-induced decrease of catalase activity, although there was no significant

Table 1
Effect of bicyclol on SOD, catalase, GR and GSH-peroxidase activity in acute alcohol-intoxicated mice

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GR (U/g protein)	GSH-peroxidase (U/mg protein)
Control	223.78±29.27	8.77±0.89	4.78±1.79	2.98±0.52
Alcohol	143.90±25.53[b]	7.14±0.68[a]	2.43±0.94[a]	1.62±0.09[b]
By 200 mg/kg	210.48±40.68[c]	6.90±0.34	3.72±1.34	3.81±0.49[d]
By 300 mg/kg	223.40±39.22[d]	7.59±1.1	3.98±0.72[c]	4.52±1.06[d]

Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 1.5 h after alcohol administration. a, $P<0.05$, b, $P<0.001$ vs. control group; c, $P<0.05$, d, $P<0.001$ vs. alcohol group.

difference compared with alcohol group. Bicyclol also showed the protection on the decreasing of GR activity, although statistical significance was only observed in 300 mg/kg group (Table 1).

3.5. Effect of bicyclol on the expression of hepatic TNF-α and IL-1β in alcohol-intoxicated mice

The over expression of liver TNF-α and IL-1β induced by alcohol was evaluated at both protein and gene levels by ELISA and RT-PCR, respectively. The results showed that hepatic TNF-α and IL-1β levels were elevated at 1.5 h after alcohol administration and reached to peak level at 12 h, approximately 2.4 and 1.7 times of control (Fig. 7). Bicyclol significantly alleviated hepatic TNF-α and IL-1β production in a dose-dependent manner (Fig. 8). The mRNA expression of TNF-α and IL-1β were increased by 2 and 2.4 times after alcohol treatment and bicyclol significantly inhibited such changes (Fig. 9). TNF-α localization was further visualized by immunohistochemistry. As shown in Fig. 12, TNF-α positive cells were predominantly Kupffer cells which localize on the sinusoid wall. Bicyclol pretreatment (300 mg/kg) can decrease

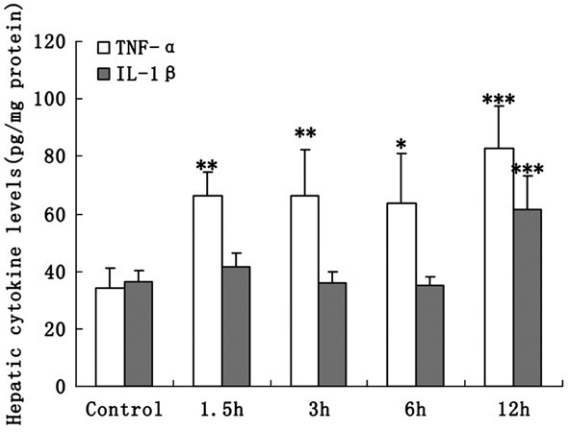

Fig. 7. Time-course changes of hepatic TNF-α and IL-1βlevels in acute alcohol-intoxicated mice. Alcohol (6 g/kg) was administered to mice by gavage. The animals were sacrificed at 1.5, 3, 6, and 12 h after alcohol administration. Data were expressed as means±SD ($n=8$). *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ vs. control group.

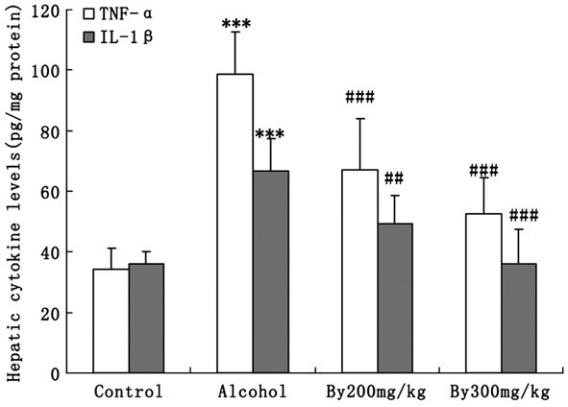

Fig. 8. Effect of bicyclol on hepatic TNF-α and IL-1βlevels in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 12 h after alcohol administration. Data were expressed as means±SD ($n=8$). ***, $P<0.001$ vs. control group; ##, $P<0.01$, ###, $P<0.001$ vs. alcohol group.

TNF-α expression on Kupffer cells, which was consistent with the results of ELISA and RT-PCR studies.

3.6. Effect of bicyclol on the elevation of plasma endotoxin in alcohol-intoxicated mice

Endotoxin is an important factor that has been demonstrated to trigger TNF-α production in alcohol-induced liver injury. It was found that plasma endotoxin level was markedly increased at 1.5 h and declined to the normal level at 6 h after alcohol

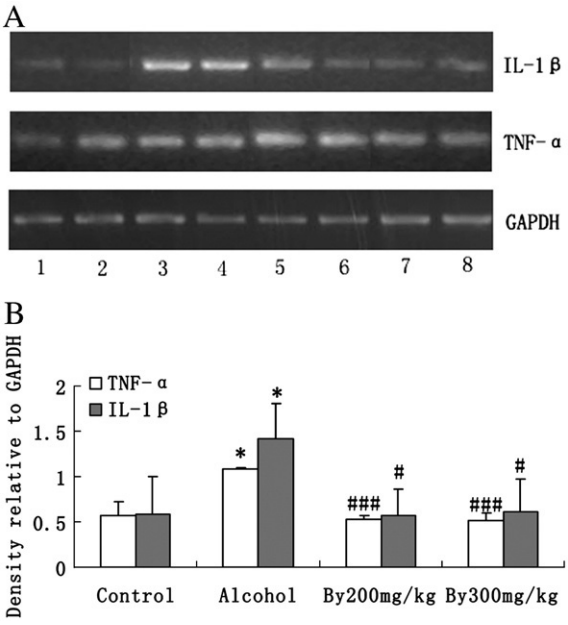

Fig. 9. Effects of bicyclol on hepatic TNF-α and IL-1βmRNA expression in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 12 h after alcohol administration. (A): lane 1–2, Control; lane 3–4, Alcohol; lane 5–6, By 200 mg/kg; lane 7–8, By 300 mg/kg. (B): Ratio of PCR products relative to GAPDH. Data were expressed as means±SD ($n=4$). *, $P<0.05$ vs. control group; #, $P<0.05$, ###, $P<0.001$ vs. alcohol group.

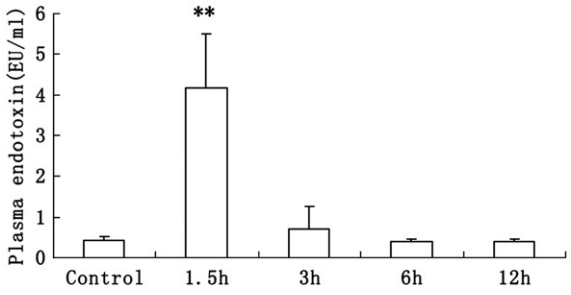

Fig. 10. Time-course changes in plasma endotoxin level in acute alcohol-intoxicated mice. Alcohol (6 g/kg) was administered to mice by gavage. The animals were sacrificed at 1.5, 3, 6, and 12 h after alcohol administration. Data were expressed as means±SD (n=6). **, P<0.01 vs. control group.

administration (Fig. 10). Pretreatment of bicyclol (200, 300 mg/kg) significantly inhibited elevation of plasma endotoxin level by 79% and 60%, respectively (Fig. 11).

3.7. Effect of bicyclol on acute alcohol-induced hepatic CD14 expression

Liver CD14 expression was determined at 12 h after alcohol administration by immunohistochemical staining. As shown in Fig. 12, alcohol resulted in a massive induction of CD14 which mainly distributed on Kupffer cells, compared with control group. Administration of bicyclol (300 mg/kg) reduced the immunoreactivity of CD14, suggesting its inhibition on CD14 up-regulation by alcohol.

4. Discussion

The results of present study demonstrated that acute alcohol administration caused liver injury as evidenced by the elevation of serum ALT and hepatic triglyceride levels, swelling and hydropic degeneration of hepatocytes, which reflected early biochemical and pathological changes in alcoholic liver disease. Bicyclol pretreatment offered significant protection to acute alcohol-intoxicated mice by attenuating ALT elevation and hepatic triglyceride accumulation in a dose-dependent manner. Histopathologcial changes induced by alcohol were also remarkably improved by bicyclol treatment.

The role of oxidative stress in the development of alcoholic liver disease has been suspected since the early 1960s by Diluzio (1964) and Diluzio and Hartman (1967), who observed that alcohol administration promoted the oxidative breakdown of cell membranes. Studies using the intragastric feeding model have demonstrated that alcohol-induced liver injury was associated with increased lipid peroxidation, formation of protein carbonyl, 1-hyroxyl ethyl radical and lipid radicals, and decrease of hepatic antioxidant defense, providing the most convincing evidence about the pathogenic role of oxidative stress (Rouach et al., 1997; French et al., 1993; Polavarapu et al., 1998; Knecht et al., 1995; Nanji et al., 1994). Our results confirmed the involvement of oxidative stress in acute alcohol-induced liver injury and showed significant protective effect of bicyclol, as evidenced by alleviation of lipid peroxidation. Furthermore, both the compromised non-enzymatic antioxidant GSH and enzymatic antioxidants, including SOD, catalase, GR and GSH-peroxidase, were restored by bicyclol treatment. In combination with previous reports concerning its capability to scavenge free radicals generated during CCl_4 activation (Liu et al., 2005), it can be concluded that the attenuation of acute alcohol-induced oxidative stress by bicyclol was due to its ability to restore the balance between generation and clearance of reactive oxygen species.

In addition to oxidative injury, abnormal cytokine metabolism is also a major feature of alcoholic liver disease (McClain et al., 2004). The expression of TNF-α and IL-1β were found to be enhanced in both animal model and patients with alcoholic liver disease (McClain et al., 1997). In particular, the critical role of TNF-α in alcoholic liver disease has been demonstrated in TNFR1 deficient mice, which were protected against alcohol-induced liver injury compared to wild type mice (Yin et al., 1999). Furthermore, neutralization of TNF-α by a specific antibody has been shown to attenuate hepatic necrosis and inflammation caused by chronic alcohol exposure (Iimuro et al., 1997). In this study, bicyclol pretreatment significantly attenuated alcohol-induced TNF-α and IL-1β expression at both protein and gene levels. Immunohistochemical staining for TNF-α showed that it was located on the membranes of Kupffer cells, which was consistent with previous study (Zhou et al., 2003). Bicyclol decreased the number of positive staining cells, further validating its effect on alcohol-induced TNF-α expression. Many reports showed that alcohol-induced reactive oxygen species can activate redox-sensitive nuclear factor (NF)-κB, which in turn led to TNF-α expression (Kono et al., 2000a,b; Wheeler et al., 2001a,b; Yin et al., 1999). In addition, numerous studies demonstrated that treatment with antioxidants, such as allopurinol, ebselen, diphenyleneiodonium sulfate attenuated NF-κB activation and TNF-α expression (Kono et al., 2000a,b, 2001a,b). Thus, we hypothesized that the inhibitory effect of bicyclol on TNF-α expression was at least in part mediated by its antioxidant property.

Activated Kupffer cells were generally considered to be responsible for the production of inflammatory cytokines. Bacterial endotoxin was found to be one of the substances that effectively activate Kupffer cells and played a critical role in

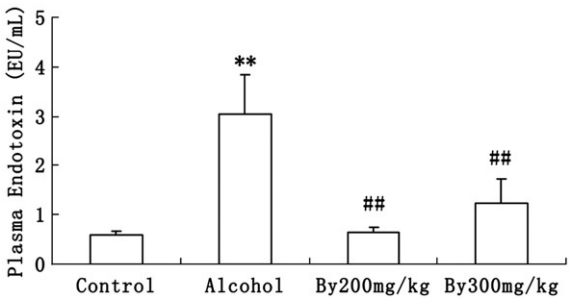

Fig.11. Effect of bicyclol on plasma endotoxin level in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice three times before alcohol treatment. Mice were sacrificed at 1.5 h after alcohol administration. Data were expressed as means±SD (n=6). **, P<0.01 vs. control group; ##, P<0.01 vs. alcohol group.

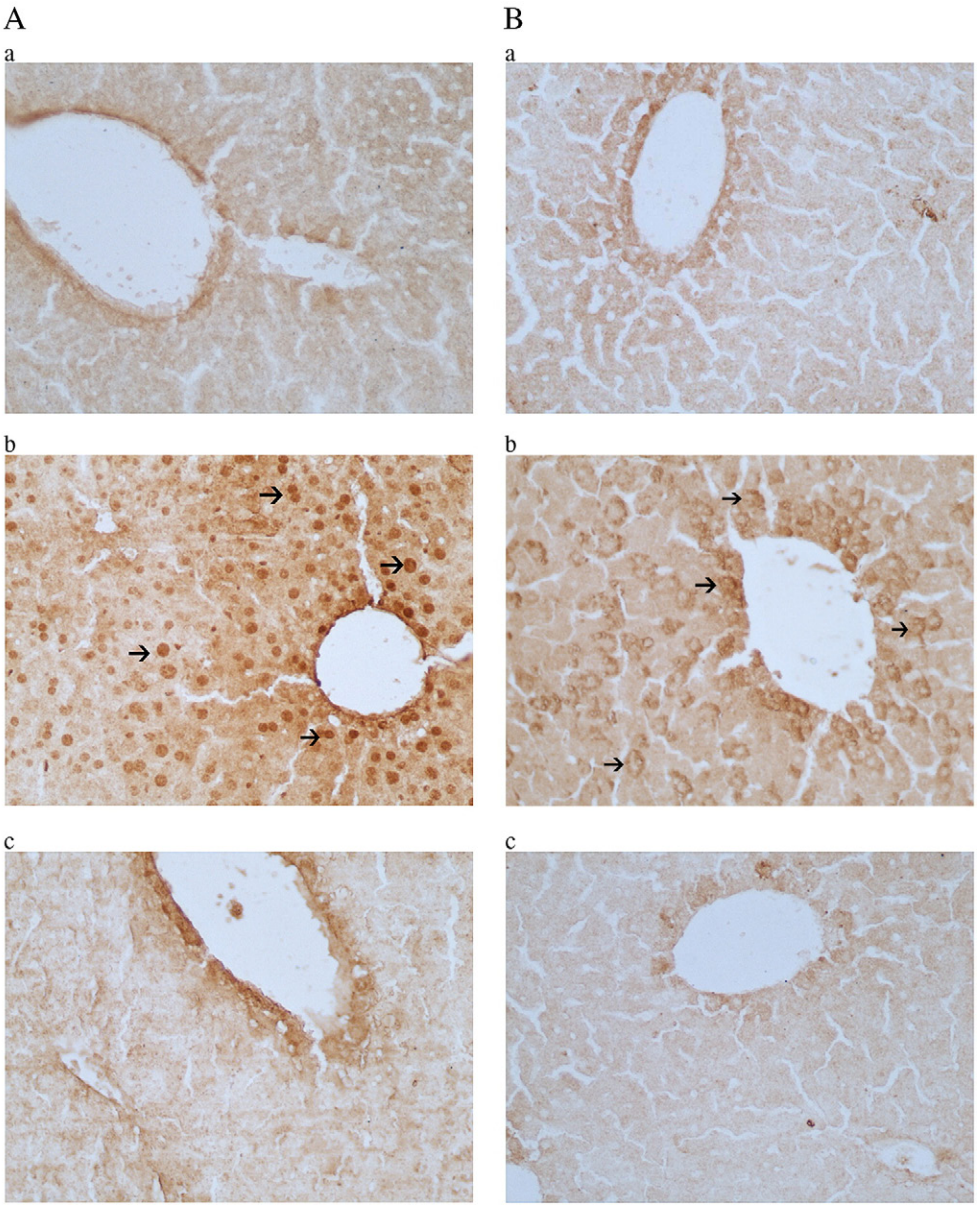

Fig. 12. Localization of liver TNF-α and CD14 expression in acute alcohol-intoxicated mice. Bicyclol (200, 300 mg/kg) was given orally to mice ($n=5$) three times before alcohol treatment. Mice were sacrificed at 12 h after alcohol administration. 1: expression of TNF-α; 2: expression of CD14. a: Control; b: Alcohol; c: Pretreatment with Bicyclol. Arrows: Positive cells. Original magnification × 100.

alcoholic liver disease. It was reported that circulating endotoxin level was elevated in patients with alcoholic liver disease (Fukui et al., 1991) and experimental models of alcoholic liver disease (Nishiyama et al., 2002; Adachi et al., 1995; Nanji et al., 1997), while eliminating endotoxin from intestine by antibiotics can completely prevent alcohol-induced liver injury. In the present study, we found that plasma endotoxin level was enhanced by alcohol, which was in line with the previous study. Bicyclol pretreatment effectively suppressed the elevation of endotoxin level, which may be related with the effect of bicyclol on increased intestinal permeability induced by alcohol. The underlying mechanisms need to be further investigated. Reduced plasma endotoxin level may result in the inhibition of Kupffer cell activation, and therefore explained the down-regulation of pro-inflammatory cytokine expression by bicyclol from another perspective.

CD14 plays a pivotal role in the activation of Kupffer cells by endotoxin, which has been demonstrated in numerous studies (Wright et al., 1990; Ferrero et al., 1993; Haziot et al., 1996). Therefore we examined liver CD14 expression by immunohistochemistry. The results showed that CD14 receptor, located on the member of Kupffer cells, was up-regulated by alcohol. Bicyclol was found to weaken the positive staining of CD14, suggesting that bicyclol may prevent Kupffer cell activation by interfering with its signal transduction in addition to decreasing its ligand endotoxin. Furthermore, the anti-oxidative property of bicyclol may contribute to its down-regulation of CD14 expression, for it has been reported that

alcohol-induced up-regulation of CD14 involved oxidant-dependent AP-1 pathway (Wheeler and Thurman, 2003).

In conclusion, bicyclol showed significant protective effect on acute alcohol-induced liver injury. The hepatoprotective action of bicyclol is most likely to be mediated by its ability to attenuate oxidative stress, suppress cytokine expression at both protein and gene level, and inhibit the activation of Kupffer cell by decreasing plasma endotoxin level and CD14 expression.

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