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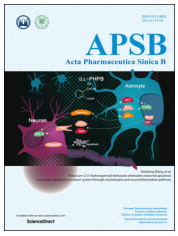

ORIGINAL ARTICLE

# *Schisandra sphenanthera* extract (Wuzhi Tablet) protects against chronic-binge and acute alcohol-induced liver injury by regulating the NRF2-ARE pathway in mice

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**KEY WORDS**

Alcoholic liver injury;
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Oxidative stress

**Abstract** Alcohol abuse leads to alcoholic liver disease and no effective therapy is currently available. Wuzhi Tablet (WZ), a preparation of extract from *Schisandra sphenanthera* that is a traditional hepato-protective herb, exerted a significant protective effect against acetaminophen-induced liver injury in our recent studies, but whether WZ can alleviate alcohol-induced toxicity remains unclear. This study aimed to investigate the contribution of WZ to alcohol-induced liver injury by using chronic-binge and acute models of alcohol feeding. The activities of ALT and AST in serum were assessed as well as the level of GSH and the activity of SOD in the liver. The expression of CYP2E1 and proteins in the NRF2-ARE signaling pathway including NRF2, GCLC, GCLM, HO-1 were measured, and the effect of WZ on NRF2 transcriptional activity was determined. We found that both models resulted in liver steatosis accompanied by increased transaminase activities, but that liver injury was significantly attenuated by WZ. WZ administration also inhibited CYP2E1 expression induced by alcohol, and elevated the level of GSH and the activity of SOD in the liver. Moreover, the NRF2-ARE signaling pathway was activated by WZ and

*Abbreviations:* ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ARE, antioxidant response element; CYP2E1, cytochrome P450 2E1 enzyme; EtOH, ethanol; GCLC, glutamate–cysteine ligase catalytic subunit; GCLM, glutamate–cysteine ligase modifier subunit; GSH, glutathione; H&E, hematoxylin and eosin; HO-1, heme oxygenase-1; NRF2, nuclear factor erythroid 2-related factor 2; SOD, superoxide dismutase; WZ, Wuzhi Tablet.

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the target genes were all upregulated. Furthermore, WZ significantly activated NRF2 transcriptional activity. Collectively, our study demonstrates that WZ protected against alcohol-induced liver injury by reducing oxidative stress and improving antioxidant defense, possibly by activating the NRF2-ARE pathway.

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## 1. Introduction

Excessive alcohol consumption with consequent liver injury has become an alarming health problem globally. Alcoholic liver disease (ALD) is associated with a spectrum of liver disorders, ranging from steatosis to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma[1,2]. Alcohol remains a major cause of morbidity and mortality in liver diseases[3,4]. Furthermore, harmful alcohol intake is ranked as one of the top five risk factors for death and disability worldwide with 2.5 million deaths and 69.4 million annual disability adjusted life years[5]. Despite the profound economic and health impact of ALD, the underlying mechanisms of alcohol-induced liver injury remain obscure and no targeted therapy is available. Therefore, developing novel, safe and pathophysiology-oriented therapies against alcohol-induced liver injury is necessary.

Accumulating evidence[6–9] reveals that oxidative stress plays a vital role in the pathogenesis of ALD. Ethanol is metabolized in the liver to acetaldehyde mainly by alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1). The expression of CYP2E1, which is responsible for generation of reactive oxygen species (ROS) to induce oxidative stress, ER stress and steatosis, has been shown to be induced in response to excessive ethanol intake[10–12]. ROS can react with and then cause damage to complex cellular molecules such as DNA and proteins, which eventually leads to hepatic injury[13]. Moreover, CYP2E1 is suggested to contribute to ethanol-induced liver injury and thus inhibition of CYP2E1 activity or CYP2E1-induced oxidative stress will alleviate ethanol toxicity[14–16].

The nuclear factor-erythroid 2-related factor 2 (NRF2), is a transcription factor locating in the cytoplasm under normal conditions. When cells are under oxidative stress, NRF2 translocates into the nucleus and initiates the transcription of a series of antioxidant and cytoprotective genes *via* the antioxidant response element (ARE)[17]. NRF2 is involved in multiple biochemical processes, including inhibiting lipogenesis, supporting $\beta$-oxidation of fatty acids, facilitating flux through the pentose phosphate pathway, and increasing NADPH regeneration[18]. As has been known, the process of ethanol metabolism produces a great amount of reactive oxygen species, directly causing cellular damage. Hence, stimulation of NRF2-ARE pathway that regulates the cellular redox status may contribute to preventing the ethanol-induced oxidative stress.

*Schisandra sphenanthera*, a Chinese traditional medicine, has been widely used in clinical practice and can protect the liver and restore liver functions[19–21]. Wuzhi Tablet (WZ), the ethanol extract of *Schisandra sphenanthera*, also has excellent hepatoprotective effects and has been used in chronic hepatitis patients in the clinic to restore liver function. Our previous study characterized the hepatoprotective effect of WZ and determined the chemical fingerprint of WZ and its six major active lignans, including schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B, and schisantherin A[22]. We determined that WZ counteracted acetaminophen (APAP)-induced hepatotoxicity by regulating the NRF2-ARE and p53/p21 pathways[19,23–25]. Also, an active component of WZ, schisandrol B, could protect against acetaminophen-induced hepatotoxicity by inhibiting CYP-mediated APAP bioactivation and regulation of p53, p21, CCND1, PCNA and BCL-2 to promote liver regeneration[26]. In addition, WZ could also facilitate liver regeneration after partial hepatectomy in mice[27]. However, the protective effect of WZ against ethanol-induced liver injury and the involvement of NRF2-ARE signaling pathway are incompletely understood.

In the current study, a chronic-binge model (a model proposed by the US National Institute on Alcohol Abuse and Alcoholism, NIAAA) and an acute model were employed, which mimics the two drinking patterns of people: the chronic drinking habit plus a single binge episode, and excessive alcohol consumption, respectively. The contribution of WZ to alcohol-induced liver injury and the related molecular pathways were investigated.

## 2. Materials and methods

### 2.1. Materials

Alcohol (95%) and sodium carboxyl methyl cellulose (CMC-Na) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Liber-DeCarli liquid diet was obtained from TROPHIC Animal Feed High-Tech Co., Ltd. (Nantong, China). Maltose dextrin was from Oddfoni Biotechnology Co., Ltd. (Nanjing, China). Wuzhi Tablets, 7.5 mg schisantherin A in one tablet, were manufactured by Fanglue Pharmaceutical Company (batch number 1510029, Guangxi, China). Antibody against CYP2E1 was purchased from Boster Biotechnology Co., Ltd. (Wuhan, China). Antibodies against NRF2 and heme oxygenase-1 (HO-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glutamate–cysteine ligase catalytic subunit (GCLC) and glutamate–cysteine ligase modifier subunit (GCLM) antibodies were obtained from Abcam (Cambridge, UK). The antibodies against GAPDH, histone

3 and all secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

### 2.2. Animals

C57BL/6 male mice, 8–10 weeks old, were bought from Sun Yat-sen University Laboratory Animal Center (Guangzhou, China). The mice were housed under a humidity of 55%–60%, temperature of 22–24 °C, a 12 h light/12 h dark cycle, and were given free access to standard diet and water. All mice were provided with proper care following the guidelines of China, and all animal procedures were reviewed and approved by the Sun Yat-sen University Ethics Committee on the Care and Use of Laboratory Animals (License: IACUC-DD-15-1002).

### 2.3. WZ preparation

Wuzhi Tablets were crushed to powder and then dissolved in 0.5% CMC-Na. An amount of 8.75, 17.5 and 35 mg powder was dissolved in 1 mL of 0.5% CMC-Na to make WZ solutions for treatment (175, 350, and 700 mg/kg). Chemical characterization data was shown in a previous study[22]. The dose of 350 mg/kg used in mice is equal to the clinical dose typically used in humans.

### 2.4. Diets, treatments, and sample collection

Mice were randomly separated into seven groups with 6–9 mice a group. In the first model the procedure was in accordance with the NIAAA model including the chronic and a single binge of ethanol feeding[28,29]. An overview of the experimental design is outlined in Fig. 1A. On days 1–5 all mice were fed with the control Lieber-DeCarli diet *ad libitum* to acclimatize to liquid diet. From day 6 the ethanol-alone group, WZ-treated group (175, 350, and 700 mg/kg, *bid.*), and the positive-control group (bicyclol, 300 mg/kg, *bid.*) were allowed free access to the Lieber-DeCarli diet containing 5% (*v*/*v*) ethanol (36% ethanol-derived calories) for 10 days. The control group and the WZ-alone control group (700 mg/kg, *bid.*) were *pair-fed* with the isocaloric control diet. On day 16, ethanol-fed and *pair-fed* mice were gavaged in the early morning with a single dose of ethanol (5 g/kg body weight) or isocaloric maltose dextrin, respectively. Mice were euthanized 9 h later.

For the acute model an overview of the experimental design is outlined in Fig. 1B. On days 1–3 mice were administrated 0.5% CMC-Na (control group), WZ (175, 350, or 700 mg/kg, *bid.* for the WZ treated group and 700 mg/kg, *bid*. for WZ alone control group), and bicyclol (positive control group, 300 mg/kg, *bid.*), respectively. On day 4, mice in the control group and the WZ-alone group were gavaged with 0.9% saline while mice in the other groups were given 6 g/kg ethanol (*bid.*). One day 5 all mice were treated following the day 4 strategy in the morning and euthanized 6 h later.

For both model, blood and liver tissues were collected, rapidly snap-frozen in liquid nitrogen, and stored at −80 °C.

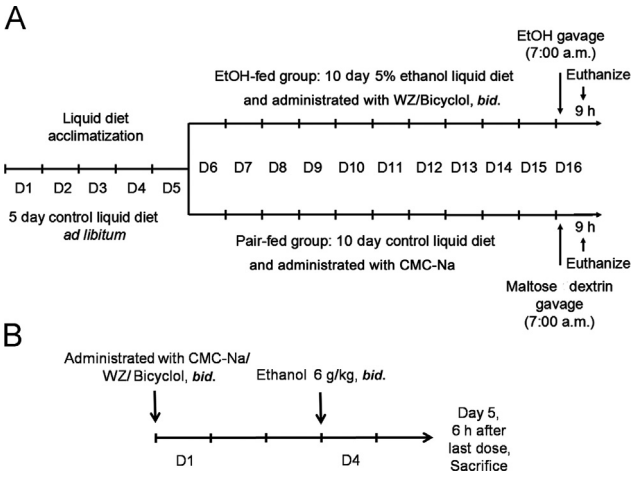


**Figure 1** The basic overview of the two model procedures. (A) Chronic-binge model (NIAAA)[28]; (B) acute model.

### 2.5. Histological analysis

A portion of liver tissue of the median and left lateral liver lobes was fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (H&E) according to a standard protocol described in our previous report[23]. The LEICA DM5000B microscope was used to examine H&E-stained liver sections (Leica, Heidelberg, Germany).

### 2.6. Biochemical assays

A kit for aspartate aminotransferase (AST) or alanine aminotransferase (ALT) assay (Kefang biotech, Guangzhou, China) and a Beckman Synchron CX5 Clinical System were used to measure the activities of serum ALT and AST to evaluate the alcohol-induced liver injury.

The activity of SOD and the levels of GSH in the liver were determined using an SOD assay kit (Nanjing Jiancheng Bioengineering Institute, China) or a GSH assay kit (Nanjing Jiancheng Bioengineering Institute, China).

### 2.7. Western blot analysis

Total and nuclear proteins were extracted from liver tissue using radioimmunoprecipitation assay (RIPA) lysis buffer and a Membrane, Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech, Shanghai, China), respectively. Total protein concentration was assessed by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA), and nuclear protein was assessed with a Bradford Protein Assay Kit (Beyotime, Jiangsu, China). Protein samples (40 μg) were loaded onto 10% and 12% SDS-PAGE gels, separated by electrophoresis and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline and then incubated with primary antibodies at 4 °C overnight followed by

corresponding secondary antibodies conjugated to horseradish peroxidase. Immunodetection was performed using an electrochemiluminescence (ECL) kit (Engreen Biosystem, Beijing, China) and analyzed with the ImageJ software (National Institute of Health, Bethesda, MD, USA).

### 2.8. Dual luciferase reporter gene assays

HEK293T cells were seeded in 96-well culture plates and incubated in a 5% $CO_2$ atmosphere at 37 °C. When about 85% confluent, cells were transfected with pEF-NRF2, pGL3-hARE reporter vectors, and pRL-TK as an internal control. After 6 h the transfected cells were treated with 0.1% DMSO, positive control sulforaphane (SFN) 5 μmol/L, WZ 31.25, 62.5, 125, 250, or 500 μg/mL for additional 24 h. Cells were then lysed for Firefly and Renilla luciferase activities assays with the Dual-Luciferase Reporter Assay System (Promega) following manufacturer's instructions.

### 2.9. Statistical analysis

Data are presented (three independent experiments or more) as mean ± SEM, and were analyzed with one-way ANOVA followed by Dunnett's multiple comparison *post hoc* test or an unpaired Student's *t*-test using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). With $P$ values $< 0.05$, data are considered statistically significant.

## 3. Results

### 3.1. WZ alleviates liver injury induced by ethanol consumption

To investigate the contribution of WZ to ethanol-induced liver injury *in vivo*, the chronic-binge (NIAAA) model and acute model were employed in which mice were fed with control/alcohol Lieber-DeCarli diet or gavaged with alcohol, respectively (Fig. 1). WZ alone had no effect on the ALT and AST activities when compared to the control group. In the chronic-binge model, ethanol treatment significantly increased the serum activities of ALT and AST 2.5- and 2-fold, respectively (Fig. 2A and B). These values were increased by 2.3- and 1.4-fold in acute model (Fig. 2C and D). However, the ethanol-induced elevation of ALT and AST activities was significantly attenuated by WZ treatment, although ALT and AST levels were still higher than control. As evidenced by H&E staining of liver sections, ethanol treatment induced marked lipid droplet accumulation, in which macrovesicular steatosis was visualized in the chronic-binge model, while microvesicular steatosis was formed in acute model. WZ treatment markedly alleviated the ethanol-induced liver steatosis in both models, the effect of which was similar to the treatment with bicyclol, the positive control drug (Fig. 2E and F). These results indicate that WZ alleviates ethanol-induced liver injury in both chronic-binge and acute model in mice.

### 3.2. WZ inhibits ethanol-induced oxidative stress

To evaluate whether WZ plays a role in reducing oxidative stress in response to ethanol intake, hepatic expression of CYP2E1, the liver level of GSH, and the activity of SOD were determined.

As shown in Fig. 3A and B, ethanol significantly upregulated the hepatic CYP2E1 protein level in both models compared to the control group. However, administration of different doses of WZ reduced CYP2E1 expression by 3%, 39%, and 42%, respectively, in the chronic-binge model. Similarly, expression was reduced by 45%, 43%, and 52%, respectively, in the acute model. Moreover, the levels of GSH and the activity of SOD in the liver were reduced following ethanol treatment in both models. However, the ethanol-induced reductions in GSH level and SOD activity were partly reversed by WZ treatment (Fig. 3C–F). Taken together, WZ inhibited ethanol-induced oxidative stress by inhibiting CYP2E1 expression and elevating the GSH level and SOD activity.

### 3.3. NRF2-ARE signaling pathway was activated by WZ in mice

We next wondered whether WZ protected against ethanol-induced oxidative stress by activating the NRF2-ARE signaling pathway to induce anti-oxidant effects. Thus, protein expression in the NRF2-ARE signaling pathway, including GCLM, GCLC, HO-1 and nuclear NRF2 levels, was examined. As shown in Fig. 4A and B, ethanol administration resulted in decreased NRF2, GCLC, GCLM, and HO-1 protein levels in the liver in both models. As expected, treatment with 350 and 700 mg/kg of WZ significantly reversed the above ethanol-induced reduction in the protein levels to normal. In addition, 350 and 700 mg/kg WZ administration increased NRF2 translocation into the nucleus as compared to the control group (Fig. 5A and B).

### 3.4. NRF2-ARE signaling pathway can be activated by WZ *in vitro*

As the involvement of the NRF2-ARE signaling pathway was demonstrated *in vivo*, it was of interest to assess the ability of WZ to regulate NRF2 transcriptional activity *in vitro*. To this end, a dual-luciferase reporter gene assay was performed. As shown in Fig. 6, treatment with the positive control drug SFN largely induced the reporter activities, indicating the successful construction of models for assessing NRF2 transactivation. Treatment with 250 and 500 μg/mL of WZ also activated the NRF2 luciferase reporter 1.8-fold ($P<0.05$) and 9-fold ($P<0.001$), respectively. These results suggest that WZ might exert an hepatoprotective effect against ethanol-induced liver injury by activating the NRF2-ARE signaling pathway.

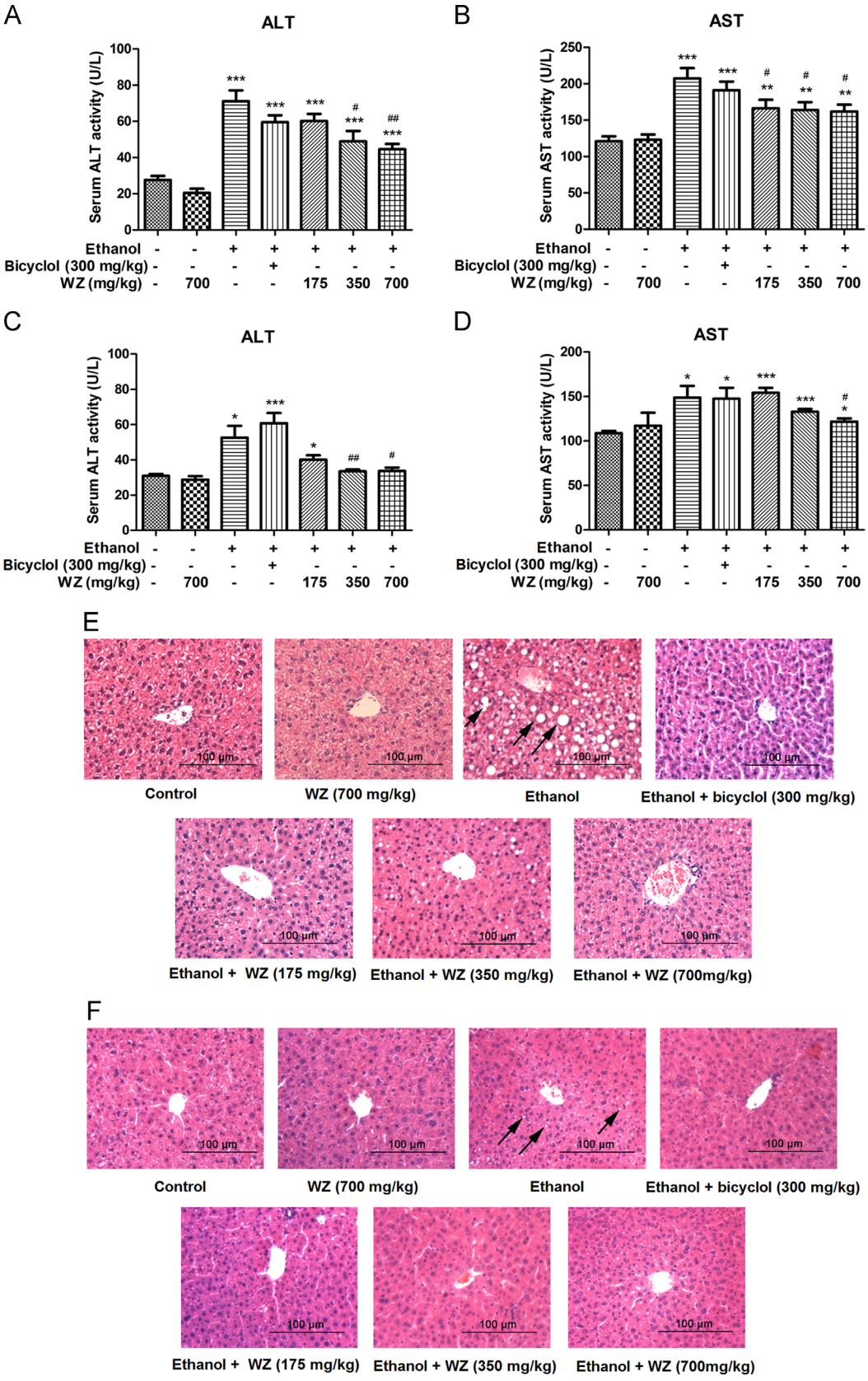


**Figure 2** Wuzhi treatment ameliorates ethanol-induced liver injury. Activities of serum ALT (A) and AST (B) in the chronic-binge model; activities of serum ALT (C) and AST (D) in the acute model. Data are presented as the mean ± SEM; $n = 6$–9. H&E-stained liver sections of chronic (E) and acute models (F), visualized at 100 ×, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* ethanol group.

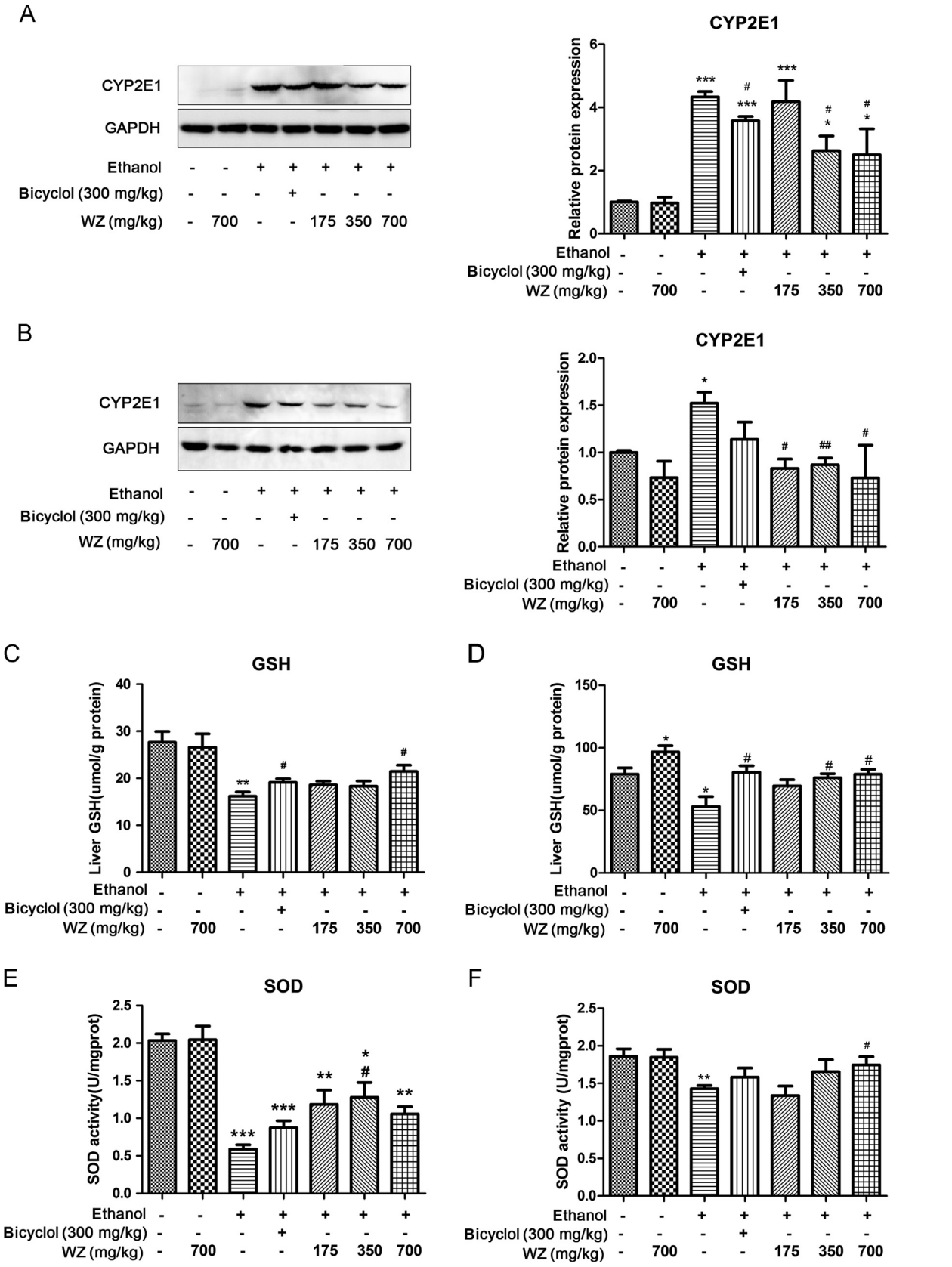


**Figure 3** Effect of WZ on ethanol-induced oxidative stress. Western blot analysis of CYP2E1 and GAPDH total protein levels in liver in chronic (A) and acute models (B). Data are presented as the mean ± SEM, $n=3$. GSH level in the liver in chronic (C) and acute model (D); SOD activity of liver chronic (E) and acute model (F). Data are presented as the mean ± SEM, $n=4$–5. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control mice; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* ethanol group.

## 4. Discussion

Chronic-binge ethanol intake causes significant liver injury[30], which is characterized by a large amount of lipid droplet accumulation and elevation in serum ALT/AST levels. In the current study we established chronic-binge and acute ethanol intake models in mice and demonstrated that the ethanol-induced pathological alteration was improved by WZ administration, indicating that WZ exerted a protective effect against ethanol-induced liver injury.

The underlying mechanisms that regulate ethanol-induced liver injury are currently unknown. One of the mainstream views suggests that oxidative stress induced by ethanol plays an essential role in liver damage[31–33]. Oxidative stress exerts various deleterious effects on hepatocytes, such as dysregulation of fatty acid synthesis and oxidation, induction of mitochondrial dysfunction, and generation of cellular stress, all of which can ultimately lead to cellular death[34,35]. Ethanol is mainly metabolized by ADH into acetaldehyde in the liver, and was further oxidized to acetate by ALDH. Another system associated with ethanol metabolism is the microsomal ethanol oxidation system (MEOS), which is based on cytochromes P450 and in particular CYP2E1[34]. Specifically, CYP2E1 metabolizes ethanol and generates ROS in this process. Meanwhile, ethanol itself can

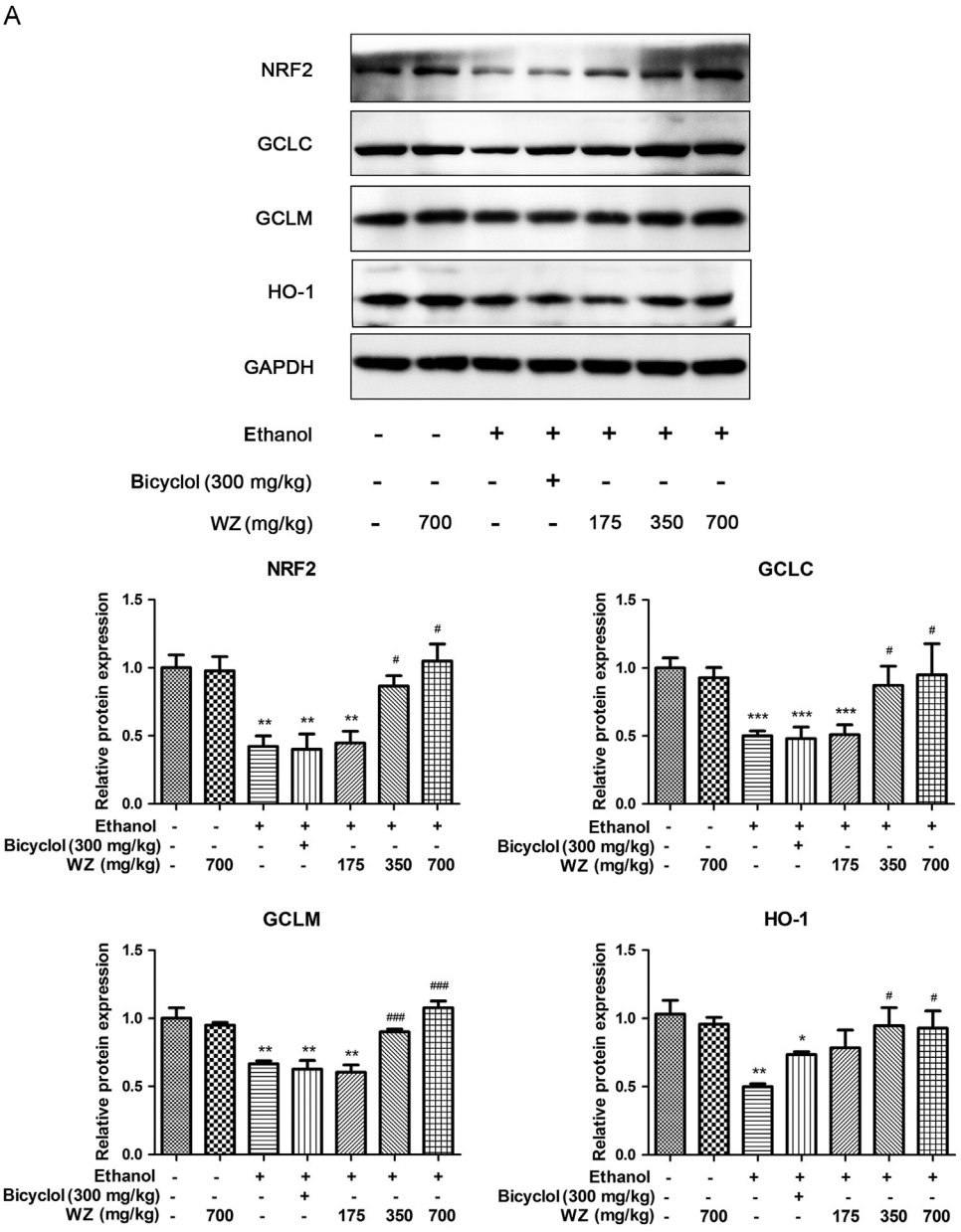


**Figure 4** Effect of WZ on protein expression of NRF2 target genes. Western blot analysis of NRF2, GCLC, GCLM, HO-1 and GAPDH total protein levels in liver from chronic (A) and acute model (B). Data are presented as the mean ± SEM, $n=3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control mice; $^{\#}P<0.05$ *versus* ethanol group.

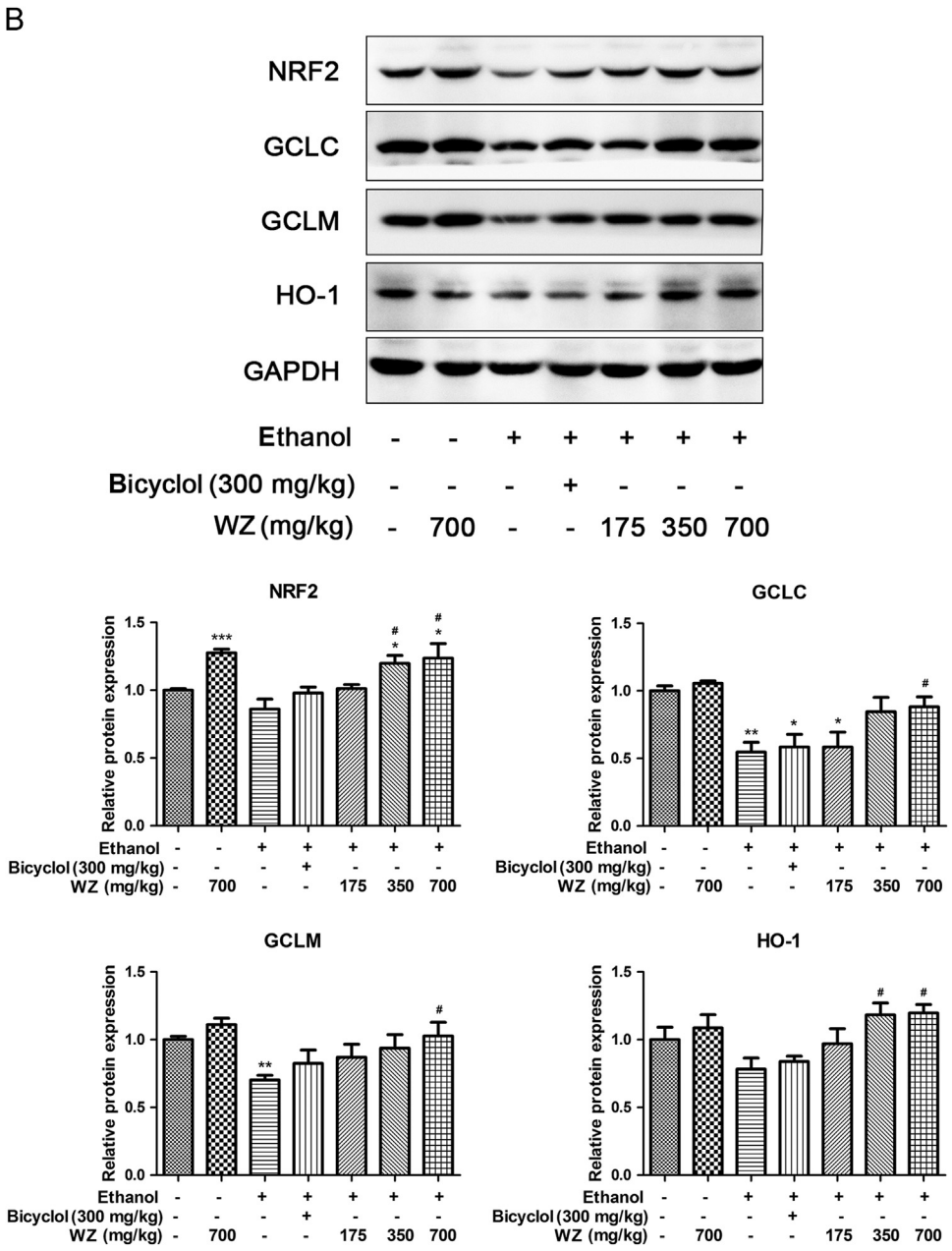


**Figure 4** (*continued*)

induce CYP2E1 expression, which further exacerbates this oxidative stress[36]. Hence inhibition of ethanol-induced expression of CYP2E1 and oxidative stress may be crucial to protect against alcoholic liver disease. In the present study, we demonstrated that WZ elevated the GSH level and SOD activity in the liver, indicating enhancement of the antioxidant defense system. Moreover, upregulated CYP2E1 expression was reversed to normal by WZ treatment, which was consistent with the results of our previous study[23], in which WZ could also inhibit CYP2E1 activity.

NRF2 is the transcription factor that regulates the expression of multiple antioxidant and cytoprotective genes against oxidative stress triggered by injury and inflammation in the body[37]. A study has found that increased HO-1 expression in the liver could alleviate chronic ethanol-induced liver injury in mice, by reducing liver cell death[38]. Our previous study demonstrated that co-administration of WZ upregulated the acetaminophen-induced reduction in protein level of NRF2, GCLC, GCLM and HO-1[23]. In accordance with the above results, 350 and 700 mg/kg WZ treatment significantly reversed the ethanol-induced reduction of NRF2, GCLC, GCLM, HO-1 protein levels to normal. We further found that 350 and 700 mg/kg WZ administration increased the NRF2 translocation into the nucleus to initiate the transcription of a series of antioxidant and cytoprotective genes. To a lesser extent, alcohol alone also increased NRF2 translocation into nucleus. In addition, we performed dual-luciferase reporter gene assays *in vitro*, the results of which coincided with the *in vivo* results. Therefore, we postulate that WZ protected the liver from alcoholic injury by regulating NRF2-ARE signaling.

Alcoholic liver disease has been identified as an important clinical issue which has a profound economic and health impact on

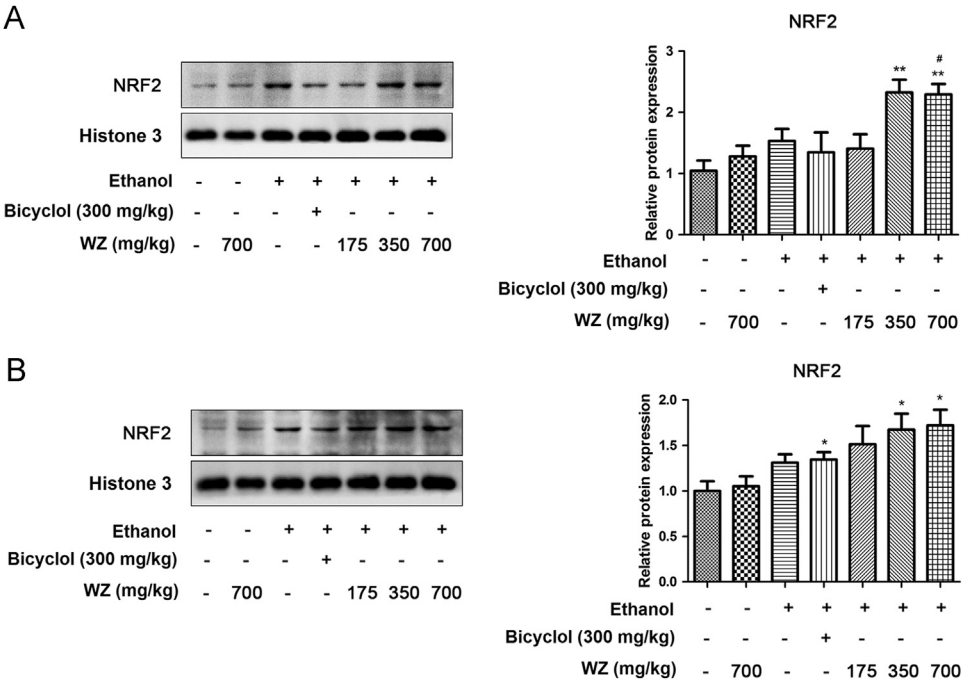


**Figure 5** The levels of NRF2 and histone 3 nuclear protein in chronic (A) and acute model (B). Data are presented as the mean ± SEM, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control mice; $^{\#}P < 0.05$ *versus* ethanol group.

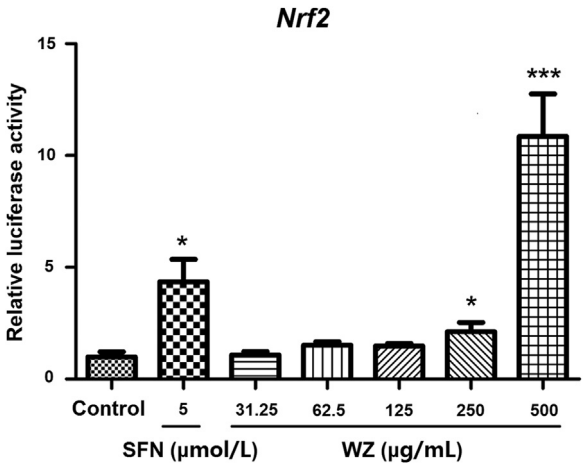


**Figure 6** WZ activates NRF2-ARE signaling pathways. Effect of WZ on NRF2 luciferase activity was measured by reporter gene assay. HEK293T cells were treated with 0.1% DMSO, sulforaphane 5 μmol/L, WZ 31.25, 62.5, 125, 250 and 500 μg/mL. Data are presented as the mean ± SEM ($n$=4–5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control mice.

patients and society. Considerable progress has been made to clarify the underlying mechanism of ALD and develop new therapeutic strategies for alcoholic liver injury, but no effective medication is available so far. WZ presents as a safe and effective drug which has already been used to restore liver function in patients with chronic hepatitis and liver dysfunction in clinical practice. Our study provides substantial evidence that WZ could also ameliorate the hepatic steatosis triggered by excessive ethanol intake and improve the antioxidant defense system.

## 5. Conclusions

Our study demonstrates that WZ protected against alcohol-induced liver injury by reducing oxidative stress and improving the antioxidant defense system, possibly through activation of the NRF2-ARE pathway. Taken together, Wuzhi Tablet might serve as an effective drug against ALD.

## Acknowledgements

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