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Original Article

4-hydroxy-2(3H)-benzoxazolone alleviates acetaminophen-induced hepatic injury by inhibiting NF-κB and activating Nrf2/HO-1 signaling pathways

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Abstract: The purpose of this study is to evaluate the protective effect of 4-hydroxy-2(3H)-benzoxazolone from *Acanthus ilicifolius* (HBAI) on acute liver injury induced by acetaminophen in mice and its mechanism. Mice were continuously treated with HBAI (200, 100, 50 mg/kg) once a day for 10 days. After that, the mice were fasted for 8 hours, followed by intraperitoneal injection of acetaminophen (300 mg/kg). The results showed that HBAI pretreatment significantly reduced acetaminophen-induced liver tissue congestion, hepatocyte apoptosis and necrosis, and inflammatory cell infiltration. HBAI could effectively reduce the levels of serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, reactive oxygen species and malondialdehyde. Interestingly, the activities of liver catalase, superoxide dismutase, glutathione and glutathione reductase were enhanced by HBAI pretreatment. Moreover, HBAI pretreatment alleviated acetaminophen-induced hepatocyte apoptosis by regulating the expression of Bcl-2 family proteins and the mitochondrial function. Further study showed that HBAI pretreatment effectively promoted the expression of Nrf2 and its signal downstream HO-1, NQO1, GCLC, GCLM, and MGST-1, suggesting the activation of the Nrf2/HO-1 signaling pathway. Meanwhile, HBAI attenuated the phosphorylation of NF-κBp65, IKKα/β, and IκBα, as well as the expression of NF-κBp50, which indicated that HBAI blocked the signal transduction of NF-κB pathway. In conclusion, HBAI protects against acetaminophen-induced acute liver injury by inhibiting the NF-κB and activating Nrf2/HO-1 signaling pathways.

Keywords: 4-hydroxy-2(3H)-benzoxazolone, acetaminophen, acute liver injury, NF-κB, Nrf2

Introduction

Liver acts as metabolic detoxification in human organs, which is vulnerable to different degrees of damage during drug metabolism. Drug-induced hepatotoxicity may have different characteristics such as inflammation, fibrosis, hepatic steatosis, oxidative stress, necrosis and apoptosis [1]. The most common damage is acute liver injury, and acetaminophen is considered as a typical drug to cause acute liver injury [2, 3].

Appropriate dose of acetaminophen is relatively safe in antipyretic and analgesic treatment and absorbed rapidly in vivo, most of which is metabolized by liver [4]. However, the intake of large doses of acetaminophen will produce free radicals in the process of liver metabolism, leading to lipid peroxidation of liver cell membrane, and the accumulation of metabolites will directly attack liver cells, resulting in continuous liver damage, liver cell necrosis and eventually liver dysfunction [5, 6]. The inherent hepatotoxicity of acetaminophen comes from its toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) in vivo. Excessive NAPQI accumulation can deplete glutathione, promoting the production of reactive oxygen species [7, 8]. When the production of reactive oxygen species exceeds the scavenging capacity of antioxidant enzymes, mitochondria will be damaged and lead to apoptosis [9]. During oxidative stress, nuclear factor erythrocyte 2 related factor 2 (Nrf2) was isolated from Kelch-like ECH-associated protein 1 (Keap1) to regulate the expression of a variety of antioxidant genes, including heme oxidase-1 (HO-1) [10,

11]. Nuclear factor κB (NF-κB) is a pleiotropic transcription factor, which also plays an important role in oxidative stress and inflammatory response [12]. It has been reported that inhibiting the activation of NF-κB and activating the Nrf2/HO-1 signal pathway may be a potential strategy to alleviate acute liver injury [13, 14].

Acanthus ilicifolius is a mangrove plant of acanthaceae, which mainly grown in the intertidal zone of southeast coastal areas of China; it widely used in folk treatment of lymph node enlargement, hepatitis, stomachache, cough, asthma [15, 16]. 4-hydroxy-2(3H)-benzoxazolone is an important pharmacological active component of *Acanthus ilicifolius*. Previous studies have shown that 4-hydroxy-2(3H)-benzoxazolone has a variety of biological effects such as anti-inflammatory and anti-oxidant. It also has a protective effect on liver fibrosis caused by carbon tetrachloride [17]. But, its effect on drug-induced liver damage remains unclear. Therefore, 4-hydroxy-2(3H)-benzoxazolone was used to treat mice to explore whether it has protective effect on acute liver injury induced by acetaminophen in this study. The study also investigated whether 4-hydroxy-2(3H)-benzoxazolone alleviates liver damage by regulating the NF-κB and Nrf2/HO-1 signaling pathways.

Material and methods

Experimental animals and reagents

Male C57BL/6J mice (6-8 weeks old and weighing 18-22 g) were required from the Hunan slack Jingda Experimental Animal Co., Ltd. (Hunan, China).

The 4-hydroxy-2(3H)-benzoxazolone (HBAI) with purity over 98% was manufactured by the Department of Pharmaceutical Chemistry of Guangxi Medical University [18]. Acetaminophen was purchased from Aladdin (Shanghai, China). Bifendate Pills were purchased from Beijing Union Pharm (Beijing, China). Two-site sandwich enzyme-linked immunosorbent assay (ELISA) for mouse reactive oxygen species (ROS) was purchased from Jianglai Biotechnology (Shanghai, China). Carmellose Sodium (CMC-Na) was purchased from Xilong Scientific (Guangzhou, China). Glutathione (GSH), glutathione peroxidase (GSH-PX), malondialdehyde (MDA), Superoxide Dismutase (SOD) and Catalase (CAT) were all purchased from Nanjing jianCheng Bioengineering Company (Nanjing, China).

Experimental design

This study was approved by the Institutional Ethics Committee of Guangxi Medical University. The male C57BL/6J mice were allowed to live in an environment with a humidity of about 70% and a constant temperature of 23 ± 2°C. Mice were randomly divided into six groups with ten mice per group: the normal group (receiving 0.6% sodium carboxymethyl cellulose solution), model group (receiving 0.6% sodium carboxymethyl cellulose solution), positive group (receiving 150 mg/kg bifendate) and HBAI pretreatment groups (receiving 200, 100, 50 mg/kg HBAI). Mice were administered by intragastric gavage for 10 continuous days. After the end of pretreatment, the normal group was given the same amount of normal saline, and all the other groups were given intraperitoneal injection of acetaminophen (300 mg/kg) [19]. Six hours later, blood was taken from the eyes of mice and liver samples were collected for subsequent experiments.

Histopathology and TUNEL staining

The liver samples that were cut from the same site of the liver were fixed in 10% formalin solution, embedded in paraffin, and cut in 5-μm-thick sections. Hematoxylin and eosin (H&E) staining was performed to observe the pathologic changes, and TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was used to analyze cell necrosis as previously described [20].

Serum biochemistry

The serum levels of total bilirubin (TBIL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by a biochemical autoanalyzer.

Liver enzymatic activity

Around 10% liver tissue was homogenized with normal saline and centrifuged with 4000 g at 4°C for 30 min. The supernatant was used to evaluate the levels of CAT, GSH, GSH-PX, SOD and MDA in liver tissue. The level of ROS in liver tissue was measured with commercially-available ELISA kit according to the manufacturer's instruction.

Table 1. The sequences of primers used for real-time quantitative PCR

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
HO-1	TGCAGGTGATGCTGACAGAGG	GGGATGAGCTAGTGCTGATCTGG
NQO1	CAGCCAATCAGCGTTCGG	CTTCATGGCGTAGTTGAATGATGTC
GCLC	AGGCTCTCTGCACCATCACTT	CTCTGGGTTGGGTCTGTGTTC
GCLM	AGTTGGAGCAGCTGTATCAGTGG	TTTAGCAAAGGCAGTCAAATCTGG
MGST-1	TTTCAGTCAACTGGTGGGCATC	AAGGCCATCAACACCTCATTGTC
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGGTGTTGAAGTCGCAGGAG

Western blotting

The total protein of liver tissue was isolated by protein extraction kit (Solarbio Biotechnology Company, Beijing, China), and the total protein concentration was determined by BCA protein detection kit (Beyotime Biotechnology Company, Shanghai, China). The protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane (Millipore, Bedford, MA, USA). After washing the membrane with 0.1% Tris buffer salt Tween 20 (TBST) for 3 times, the membrane was incubated with the corresponding primary antibodies overnight at 4°C: NF-κBp65, p-NF-κBp65, NF-κBp50, IκBα, p-IκBα, IKKα/β, p-IKKα/β, Nrf2 (Cell Signaling Technology, Danvers, MA, United States); GAPDH, HO-1, Keap1, Bax, Bcl-2 (Wuhan Sanying Biotechnology Company, Wuhan, China) and Cyt C (Beyotime Biotechnology Company, Shanghai, China). After washing with TPST for 3 times, the membrane was incubated with the secondary anti-goat antibody (Santa Cruz Biotechnology Company, California, USA) in a shaking table at room temperature for one hour. Eventually, the membrane was washed again and the protein band analysis was carried out by enhanced chemiluminescence detection system (Millipore, Billerica, MA, United States).

Real-time quantitative PCR

Total RNA was extracted from liver tissue samples with Trizol reagent (Invitrogen of Carlsbad, California, USA). According to the manufacturer's instructions, the main script RT kit (Takara, Kyoto, Japan) was used for cDNA synthesis. The expression of mRNA was estimated by using 7300 RT-PCR detection system (Applied Biosystems Company, Foster City, California, USA) and SYBR Green I (Takara, Kyoto, Japan). GAPDH was an internal control for measuring the expression of related genes. **Table 1** Primer sequences used in the study.

Statistical analysis

Statistical analysis was conducted using *SPSS* 22.0 (*SPSS*, Chicago, IL, USA). One-way variance (*ANOVA*) was used to analyze the differences between the groups, and Tukey's post-hoc multiple comparison test was used to compare them at the same time. All experimental results were expressed as mean ± standard deviation (*S.D.*), *P* value less than 0.05 was considered statistically significant.

Results

HBAI could lessen the acute liver injury caused by acetaminophen

In order to evaluate the protective effect of HBAI on acetaminophen-induced acute liver injury, the liver morphology was firstly observed. The liver in the normal group had ruddy appearance, smooth surface coating, good elasticity and normal size and shape (**Figure 1A1**). The liver of the model group showed rough surface coating and low elasticity, and there were extravasated blood and punctate spots of different sizes and colors on the tissues (**Figure 1A2**); however, these conditions were ameliorated by HBAI in a dose-dependent manner (**Figure 1A4-6**).

The histopathological changes were further observed by H&E staining. As shown in **Figure 1B**, there were no obvious degeneration or necrosis or any other pathological changes in normal group, and its hepatic lobule had a clear structure; the hepatocytes were arranged orderly with the same size (**Figure 1B1**). Nevertheless, the hepatic tissue in the model group was infiltrated by inflammatory cells; the liver cords were disordered; the hepatocytes were swollen to varying degrees, accompanied by focal necrosis (**Figure 1B2**). Intriguingly, HBAI and bifendate treatment could reduce the degree of liver cell damage, suggesting the liver was effectively protected (**Figure 1A3-6** and **1B3-6**). Overall, these results indicated that HBAI could alleviate the symptoms of liver injury.

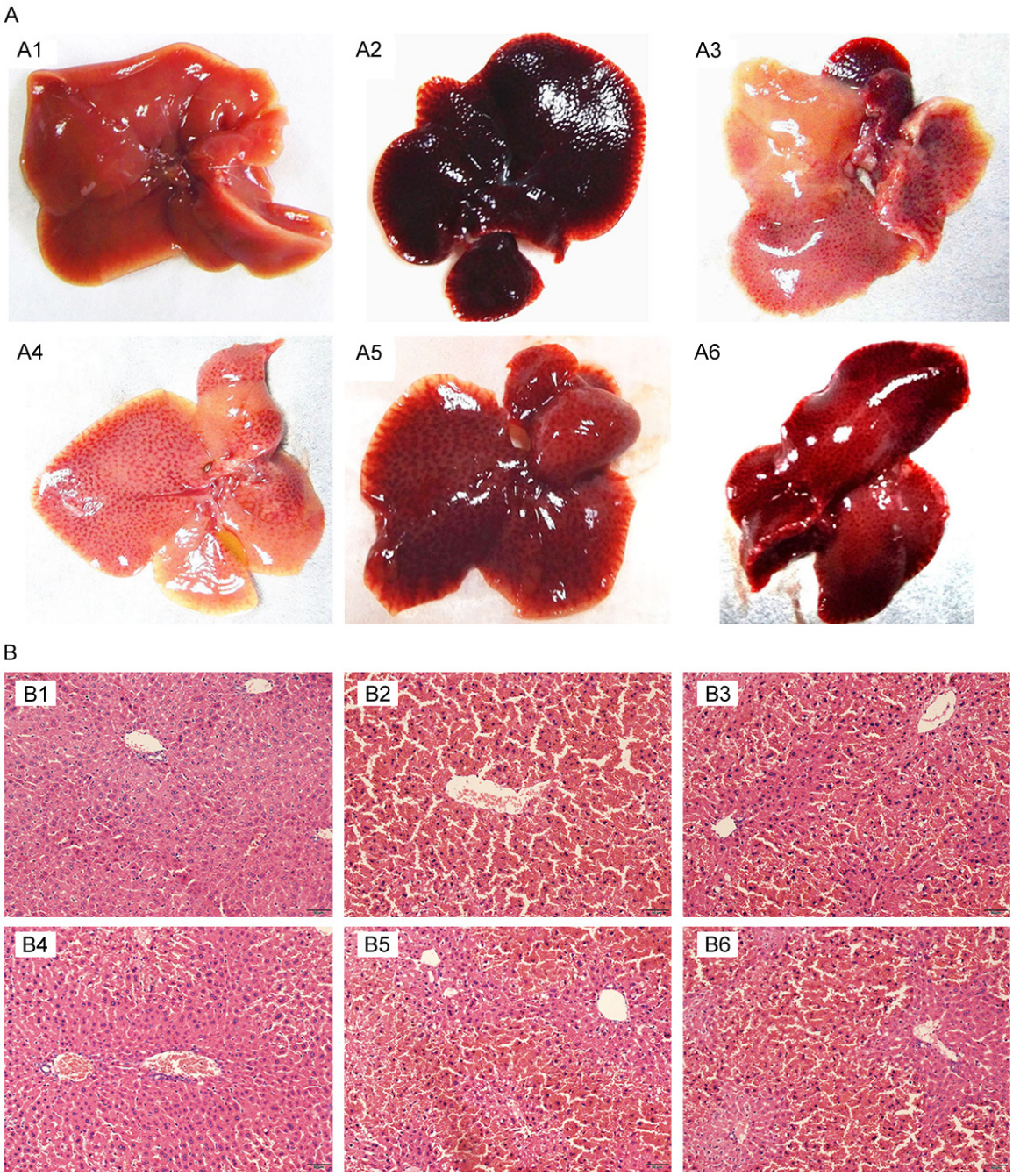

Figure 1. Pretreatment with HBAI alleviated hepatic damage caused by acetaminophen. The typical images were selected from each experiment group (n = 10). (A1-6) in (A) and (B1-6) in (B) represented the normal, model, positive, high-, medium-, and low-dose of HBAI groups. (A) The appearance of liver tissue. (B) Liver histology was observed by H&E staining (× 200).

HBAI reduced serum TBIL, ALT and AST activities

Pretreatment with HBAI protected against acetaminophen-induced hepatotoxicity in mice. As shown in **Figure 2**, the results showed that the serum levels of TBIL, ALT and AST in the model control group were markedly increased, but were significantly decreased by HBAI treatment, suggesting that HBAI could reduce liver damage by reducing the activity of total bilirubin and transaminases.

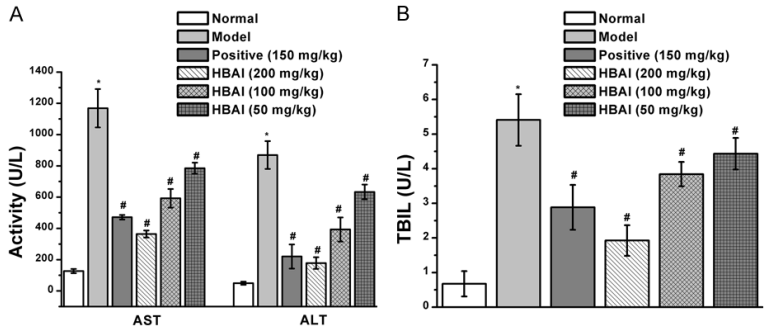

Figure 2. Pretreatment with HBAI suppressed the activity of TBIL, ALT and AST in acetaminophen-induced acute liver injury. The experimental data were all expressed as mean ± SD (n = 10). $^{*}P < 0.05$ *vs.* the normal group; $^{\#}P < 0.05$ *vs.* the model group.

HBAI alleviated oxidative stress in liver tissue

As shown in **Figure 3**, a rapid increase was found in the levels of ROS and MDA in liver tissue of the model group, while the levels of SOD, CAT, GSH and GSH-PX were decreased greatly. Compared with the model group, HBAI exerted an inhibitory effect on the activity of ROS and MDA, and increased the levels of SOD, CAT, GSH and GSH-PX. These data suggested that HBAI could suppress oxidative stress and recruit the anti-oxidative system, ameliorating oxidative damage.

HBAI mitigated apoptosis and mitochondrial dysfunction

TUNEL staining was used to indicate apoptosis in liver tissue samples. There were little apoptotic cells in the normal liver tissue (**Figure 4A1**). After acetaminophen treatment, more apoptotic cells could be seen in the model group, which was significantly different from the normal group (**Figure 4A2**). Compared with the model group, the proportion of TUNEL positive region in HBAI pretreatment group was significantly reduced (**Figure 4A4-6**). To further explain the effect of HBAI on acetaminophen-induced apoptosis, the expressions of Bcl-2 family proteins were also tested. The result showed that the expression of Bcl-2 in the HBAI pretreatment groups was significantly increased, while the expression of Bax protein was decreased (**Figure 4B**). Moreover, our result also showed that the expression of Cyt C in model group was increased after acetaminophen challenge, and this abnormal change was reversed by HBAI pretreatment, suggesting the protective effect of HBAI on mitocondria. Taken together, these data demonstrated that HBAI alleviated hepatocyte apoptosis through regulating Bcl-2 family proteins expression and improving the mitochondrial function.

HBAI activated the Nrf2/HO-1 signaling pathway

As shown in **Figure 5A**, acetaminophen significantly attenuated the expression of protein Nrf2, keap1 and HO-1. While HBAI pretreatment effectively increased the expression of these proteins. Similarly, HBAI promoted gene transcription of genes downstream of Nrf2 (**Figure 5B**). Therefore, these data indicated that the hepatoprotective effect of HBAI may be related to the activation of the Nrf2/HO-1 signaling pathway.

HBAI inhibited NF-κB activation

Due to the vital role of NF-κB activation in inflammatory, the present study further investigated the NF-κB pathway. In the **Figure 6**, the result showed that acetaminophen treatment led to a significant increase in the phosphorylation of IKKα/β, NF-κBp65 and IκBα, whereas HBAI decreased these phosphorylation levels, suggesting that HBAI inhibited the activation of the NF-κB pathway.

Discussion

The current study revealed that HBAI has significant protective effect on the liver injury caused by acetaminophen as evidenced by the improvement of histopathological changes, which provided the direct evidence for the treatment of HBAI on acetaminophen-induced toxicity. When liver injury occurred, the permeability of hepatocyte membrane was increased; glutamic oxaloacetic transaminase and glutamic pyruvic transaminase were released into the blood, suggesting that elevated levels of transaminase were an important signal of liver damage [21]. High total bilirubin was also an abnormal indicator of inflammation, necrosis and toxicity in the liver. The study

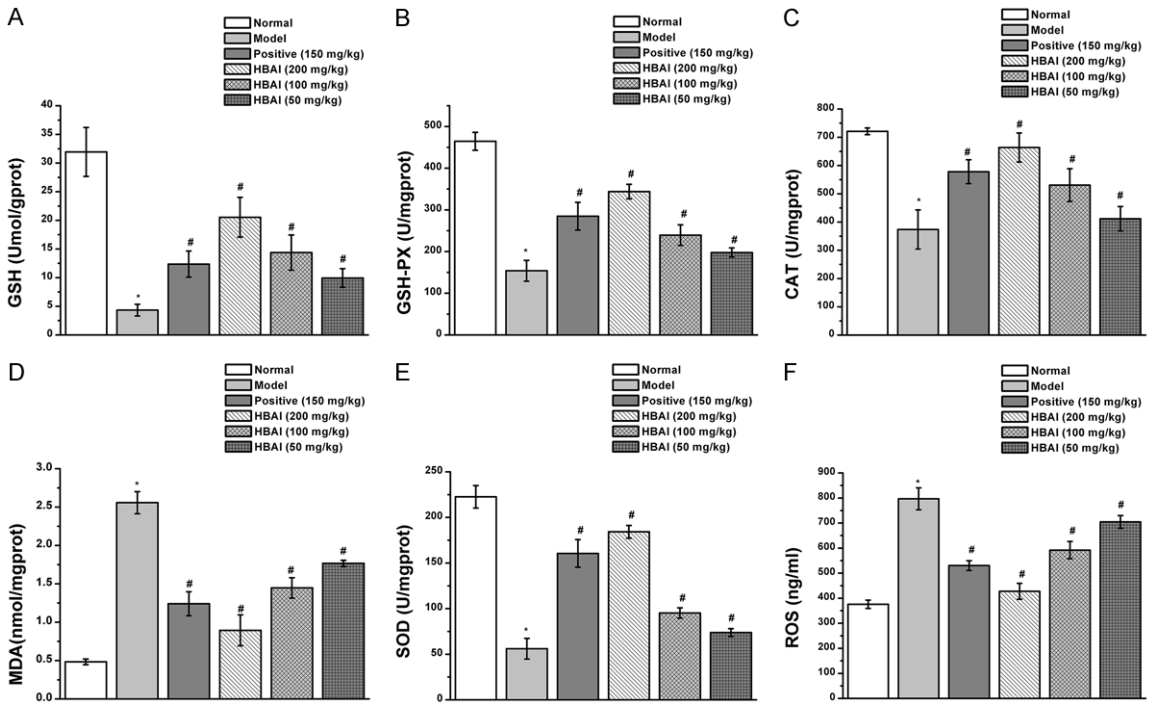

Figure 3. Pretreatment with HBAI alleviated oxidative stress induced by acetaminophen. The experimental data were all expressed as mean ± SD (n = 10). $^{*}P < 0.05$ *vs.* the normal group; $^{\#}P < 0.05$ *vs.* the model group.

showed that HBAI pretreatment protected mice from the effects of acetaminophen hepatotoxicity by refraining the levels of serum TBIL, AST and ALT.

Oxidative stress contributes to the pathogenesis and progress of liver diseases, which can lead to lipid peroxidation [22, 23]. It has been reported that glutathione and glutathione peroxidase can scavenge free radicals produced by active metabolites, protecting liver from oxidative stress [24]. Catalase and superoxide dismutase are the important antioxidant enzymes in oxidative stress, which can effectively remove reactive oxygen species. Malondialdehyde is one of the important products of lipid peroxidation, which is also involved in oxidative stress and exacerbates the progression of liver injury [25]. It has been found that reactive oxygen species (ROS) plays a central role in cell signaling, mitochondrial damage and the apoptosis mediated by death receptors. When the content of reactive oxygen species in cells is too high, it will cause damage to the body, leading to hepatocyte apoptosis [26]. In the present study, acetaminophen stimulation caused a significant decrease in the SOD, CAT, GSH and GSH-PX, but led to an increase in MDA and ROS. However, pretreatment with HBAI could reverse these abnormal changes induced by acetaminophen, indicating that HBAI ameliorated liver injury partially due to its antioxidant capacity.

A growing body of evidence suggested that acute liver injury was associated with apoptosis. Cell apoptosis is a kind of terminal pathway of cell death, which is a typical form of programmed cell death [27]. In the present study, apoptosis of liver cells was observed by TUNEL staining. The results showed that the apoptosis of hepatocytes was serious in the acute liver injury induced by acetaminophen, while the apoptosis was reduced in the HBAI pretreatment group. Apoptosis is regulated by death receptor-mediated pathways and mitochondrial pathways [28]. Bcl-2 family are important apoptotic proteins in the death receptor-mediated pathways. Bax is one of the most important pro-apoptotic genes, and the encoded Bax protein can form heterodimer with Bcl-2 and exert inhibitory effect on Bcl-2 [29]. Bcl-2 is a crucial protein to inhibit apoptosis. The increased in expression of Bcl-2 can inhibit mitochondrial permeability changes and affect the formation of giant pores, thereby

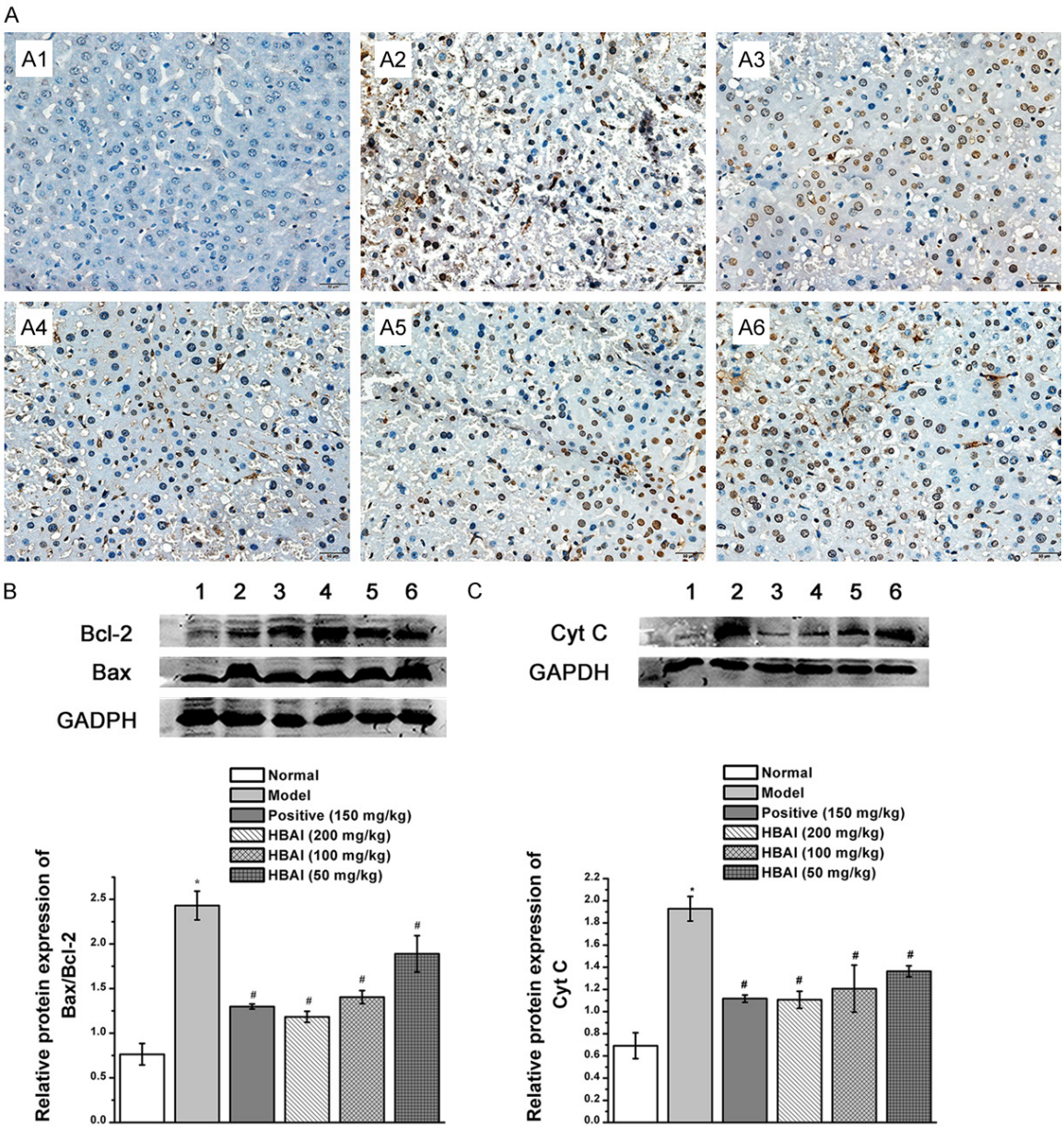

Figure 4. Pretreatment with HBAI could decrease the apoptosis and mitochondrial damage. (A1-6) in (A) and 1 to 6 in (B or C) represent the normal, model, positive, high-, medium-, and low-dose of HBAI groups. (A) The results of TUNEL staining. (B) Western bolt analysis of Bax and Bcl-2; (C) quantification of Cyt C expression based on GAPDH. The experimental data were all expressed as mean ± SD (n = 3). $^{*}P < 0.05$ *vs.* the normal group; $^{\#}P < 0.05$ *vs.* the model group.

inhibiting apoptosis [30]. The ratio relationship between Bax/Bcl-2 proteins is a key factor that determines the inhibitory effect on apoptosis. In addition, the release of cytochrome C from mitochondria is a key step in apoptosis [31]. Cytochrome C (Cyt C) was regarded as an important electron transporter in respiratory chain, which was easy to cause mitochondrial dysfunction and nuclear deoxyribonucleic acid damage [32]. Our study showed that HBAI pretreatment could increase the expression of Bcl-2 and decrease the expression of Bax. Moreover, HBAI pretreatment significantly inhibited Cyt C release from mitochondria into cytoplasm. These data suggested that HBAI alleviated hepatocyte apoptosis by regulating the Bcl-2 family protein expression and protecting the mitochondrial function.

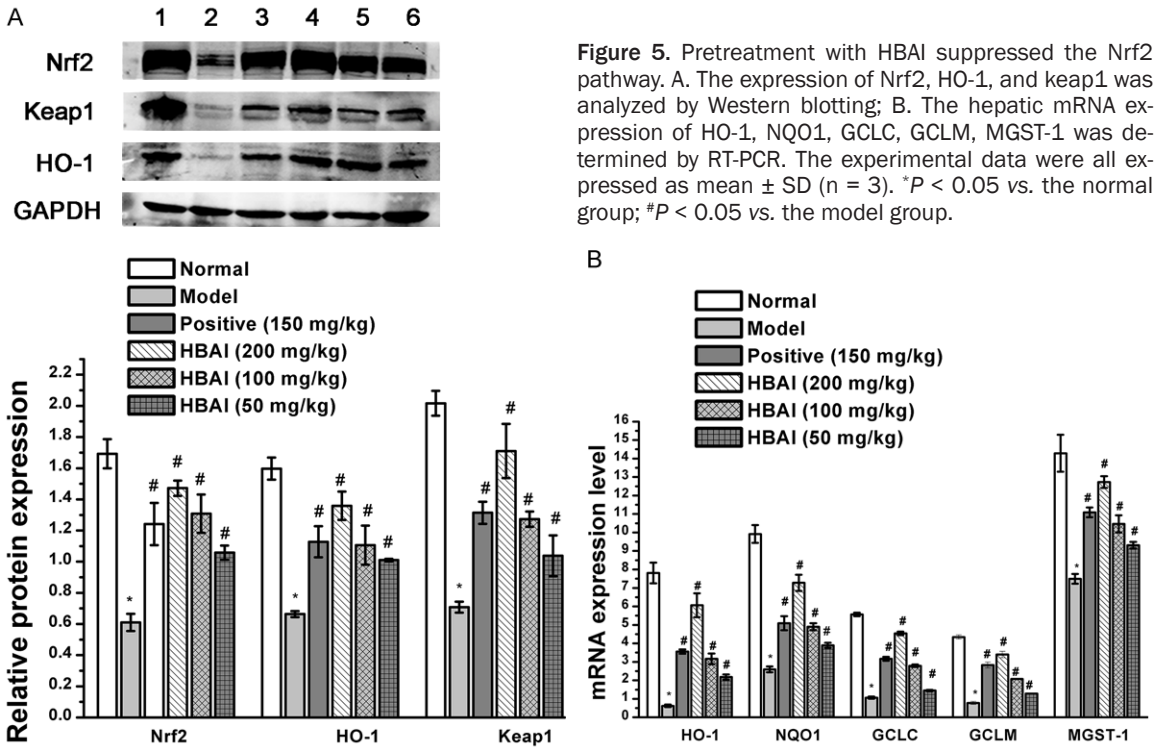

Figure 5. Pretreatment with HBAI suppressed the Nrf2 pathway. A. The expression of Nrf2, HO-1, and keap1 was analyzed by Western blotting; B. The hepatic mRNA expression of HO-1, NQO1, GCLC, GCLM, MGST-1 was determined by RT-PCR. The experimental data were all expressed as mean ± SD (n = 3). $^{*}P < 0.05$ *vs.* the normal group; $^{\#}P < 0.05$ *vs.* the model group.

In order to explore the antioxidant mechanism of HBAI, the Nrf2 pathway was further detected. Nrf2 is a basic region leucine zipper transcription factor, which can transcriptionally regulate the expression of a series of antioxidant genes and maintain the oxidation-reduction homeostasis of the liver [33, 34]. Generally, Nrf2 binds to Keap1 and exists in cells under normal conditions. In the event of oxidative stress, the cysteine residue of Keap1 is modified to change the conformation, resulting in the release of Nrf2 into the nucleus, and promoting the expression of target genes [35]. Glutamate cysteine ligase (GCL) is one of downstream genes in the Nrf2 pathway, which is composed of subunit (GCLC) and modified subunit (GCLM). It regulates the hepatotoxicity of acetaminophen by catalyzing the speed-limiting step of glutathione biosynthetic [36]. As one of the potential therapeutic targets for liver protection, HO-1 is responsible for heme catabolism of drug-metabolizing enzymes that protect cells from oxidative damage [37]. And NQO1 is act as an enzyme with antioxidant properties, which can reduce liver damage by catalyzing the reduction of quinones in cells [38]. In this study, pretreatment with HBAI could up-regulate Nrf2 and its downstream signal expression to some extent, indicating that HBAI alleviated oxidative stress by stimulating the Nrf2 pathway.

It is well known that the NF-κB pathway plays an important role in inflammation response [39]. NF-κB belongs to Rel protein family, which is recognized as an important transcriptional regulator. The regulation of NF-κB is mainly through three aspects: inhibiting its phosphorylation, blocking its nuclear localization and binding to DNA, and inhibiting the expression of target genes [40]. IKK is a serine specific protein kinase, which can be activated by extracellular stimulation and catalyze phosphorylation of specific sites of IκB (the inhibitory protein of NF-κB), leading to ubiquitination of IκB and degradation [41]. The activation of IKK will directly affect the phosphorylation of κB protein and NF-κBp65, thereby destroying the complex of IκB and NF-κB [42]. The released NF-κB enters the nucleus and initiates transcription of the regulated gene. And IκBα is an crutial member of the IκB family, phosphorylated IκBα can be degraded by ubiquitin proteasome to enhance the activation of NF-κB [43]. In this experiment, we found that HBAI could inhibit the expression phosphorylation of NF-κBp65, IκBα and IKKα/β, which

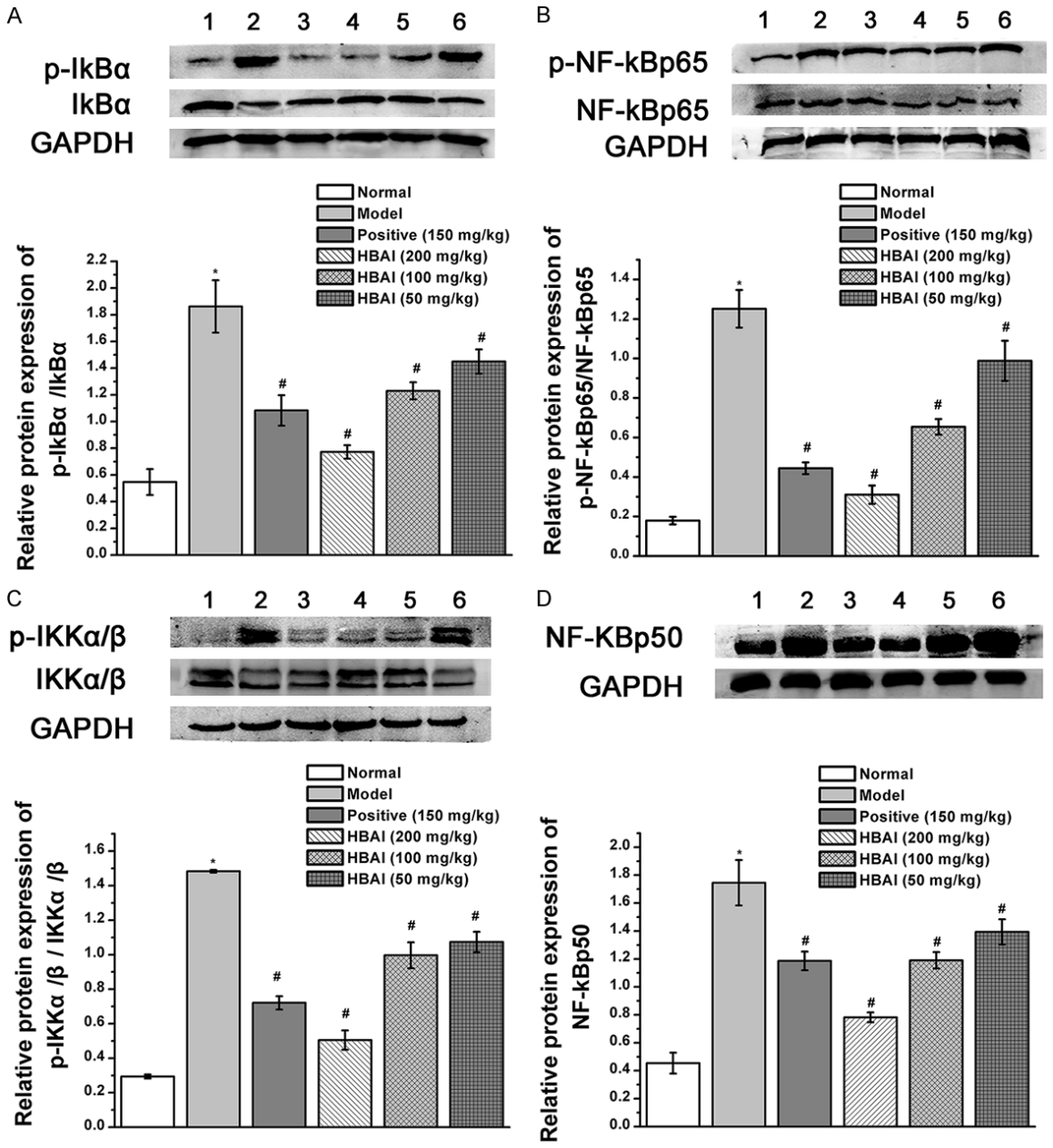

Figure 6. Pretreatment with HBAI restrained the activation of NF-κB pathway. Protein levels were measured by western blot, bands 1 to 6 represent the normal, model, Positive, high-, medium-, and low-dose of HBAI groups. The experimental data were all expressed as mean ± SD (n = 3). $^{*}P < 0.05$ *vs.* the normal group; $^{\#}P < 0.05$ *vs.* the model group.

indicated that HBAI ameliorated acetaminophen-induced inflammation by restraining the activation of the NF-κB pathway.

In summary, this study clearly demonstrates that HBAI has strong protective effect on acetaminophen-induced acute liver injury in mice, and the underlying mechanism may be related to eliminating reactive oxygen species free radicals, inhibiting the activation of NF-κB pathway, and promoting the Nrf2/HO-1 pathway (**Figure 7**).

Acknowledgements

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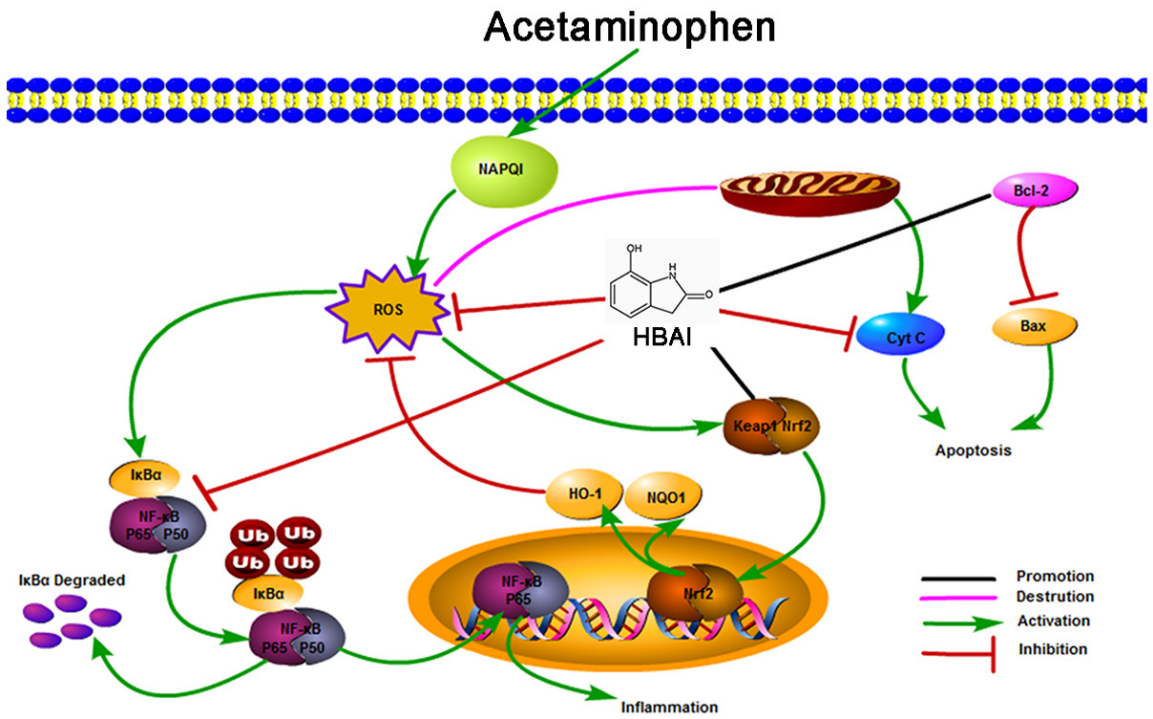

Figure 7. The schematic diagram showed the mechanism of HBAI improving acute liver injury induced by acetaminophen by inhibiting NF-κB and activating the Nrf2/HO-1 signaling pathways.

Disclosure of conflict of interest

None.

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