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# 4-hydroxybenzo[d]oxazol-2(3H)-one ameliorates LPS/D-GalN-induced acute liver injury by inhibiting TLR4/NF-κB and MAPK signaling pathways in mice

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ABSTRACT

The purpose of this study was to synthesize 4-hydroxybenzo[*d*]oxazol-2(3H)-one (HBO) and to investigate its protective effects on lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute liver injury. HBO ($C_7H_5O_3N$) was synthesized based on 2-nitro-resorcinol and identified by physicochemical analysis. In the animal experiment, mice were pretreated with HBO (50, 100, 200 mg/kg) for 10 days. At the end of pretreatment, the animals were injected with LPS (10 μg/kg)/D-GalN (700 mg/kg). The results showed that HBO significantly alleviated liver injury induced by LPS/D-GalN in mice. It remarkably decreased inflammatory response by reducing the levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Moreover, HBO notably attenuated hepatocyte apoptosis by inhibiting the release of Cytochrome C (Cyt C) from mitochondria into the cytoplasm and regulating the expression of B-cell lymphoma-2 (Bcl-2) family. Furthermore, the result showed that HBO inhibited the expressions of nuclear factor kappa-B p50 (NF-κBp50), toll-like receptor 4 (TLR4), and myeloid differentiation factor 88 (MyD88), as well as the phosphorylation of inhibitor of nuclear factor kappa-B (IκB), inhibitor of nuclear factor kappa-B kinase-α/β (IKK-α/β), nuclear factor kappa-B p65 (NF-κBp65), suggesting that HBO had a certain influence on the TLR4/NF-κB pathway. In addition, the mitogen-activated protein kinase (MAPK) signaling pathway was also affected by HBO, as evidenced by the decrease in the phosphorylation levels of extracellular regulated protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38). In conclusion, our study suggested that HBO could protect against LPS/D-GalN-induced liver injury, moreover, treatment with HBO appeared to be capable of further regulating the TLR4/NF-κB and MAPK signaling pathways.

## 1. Introduction

Liver injury is a severe syndrome, which can lead to high mortality. It can be induced by many risk factors such as viruses, toxins, drugs, and alcohol. Lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute liver injury has been widely recognized as a model to evaluate the efficiency of hepatoprotective agents [1]. Many experimental studies have confirmed that LPS/D-GalN-induced acute liver injury leads to hepatocyte apoptosis, large areas of leukocyte infiltration, and the production of proinflammatory cytokines [2,3]. It is well accepted that the inflammatory response is the most critical factor in the progression of this acute liver injury model [4]. Therefore, reducing inflammatory cytokines may be an effective way for the treatment of the acute liver injury.

The previous study showed that various signaling pathways such as Mitogen-activated protein kinase (MAPK) and Nuclear factor-κB (NF-κB) could be activated by inflammatory cytokine like TNF-α [5]. In addition, some studies have reported that LPS can bind to Toll-like receptor 4 (TLR4) of the cell membrane; afterward, TLR4 can activate many intracellular signaling pathways such as NF-κB and MAPK [6,7]. Furthermore, the activated NF-κB and MAPKs pathways in turn lead to further inflammation responses and more production of pro-inflammatory cytokines [8]. Thus, inhibiting the TLR4/NF-κB and MAPK signaling pathways should be a potential strategy to alleviate inflammatory response.

The effects of HBO on LPS/D-GalN-induced acute liver injury have

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not been investigated, and its potential mechanisms remain unclear so far. Thus, the present study aimed to explore whether HBO could alleviate LPS/D-GalN-induced acute liver injury. Additionally, TLR4/NF-κB and MAPK pathways are closely related to this model, therefore, the study investigated whether HBO could regulate TLR4/NF-κB and MAPK pathways.

Bifendate Pills are commonly used for the elevation of ALT caused by the chemical poisons and drugs, and several studies demonstrated the beneficial role of Bifendate in the protection from hepatitis and liver injury [9]. Therefore, based on its beneficial protective effects on the liver, Bifendate was chosen as the positive control drug in this study.

## 2. Materials and methods

### 2.1. Materials

D-GalN and LPS were purchased from Sigma-Aldrich (St. Louis, MO). Bifendate, a positive control drug, was supplied by Beijing Union Pharm (Beijing, China). Mitochondria Isolation Kit was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) were purchased from Shanghai Zhicheng Biological Technology Co., Ltd. (Shanghai, China). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were provided by Elabscience Biotechnology Co., Ltd. (Wuhan, China). Glutathione (GSH), Glutathione peroxidase (GSH-PX), Superoxide dismutase (SOD), Malondialdehyde (MDA), Catalase (CAT), Nitric oxide (NO), Inducible nitric oxide synthase (iNOS) and bicinchoninic acid (BCA) assay kits were produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### 2.2. Preparation of HBO

HBO ($C_7H_5O_3N$, purity > 98%) was synthesized in our Pharmaceutical Chemistry Laboratory (Guangxi Medical University, Guangxi, China) (Fig. 1). In brief, a mixture of 8.0 g $FeCl_3{\cdot}6H_2O$ and 18 g activated carbon using methanol as solvent was heated for 30 min under reflux, and then the 2-nitro-resorcinol (62.0 g, 0.4 mol) was poured into the mixture. Then 1.5 mol hydrazine hydrate (75%, 100 ml) was added by dripping slowly, and the reaction mixture was subjected to reflux for 4–5 h; thereafter, the heating was stopped and let the mixture cool slightly. The activated carbon was removed by filtration, and the methanol was removed by evaporation under reduced pressure; then a dark brown viscous liquid was obtained. N-butyl acetate (620 ml) was added to the above viscous liquid; subsequently, the urea (144 g, 2.4 mol) was added when the mixture was heated to about 90 °C. Now, the mixture was refluxed for 5 h with stirring. The reaction mixture was vacuum evaporated to remove n-butyl acetate. Next, the solid substance was washed twice by adding 500 ml water each time, stirring, heating, holding for 30 min at 80–90 °C. At that moment, the filter cake was obtained by filtering. The obtained solid was added into n-butanol twice (800 ml × 2) refluxed, and the solution was filtered when it was still hot. Afterward, the filtrate was combined, and the impurities were removed by 200 ml of water under decompression. After filtrating and drying, the brown product was got, and it was recrystallized from ethanol. The product was identified on the basis of physicochemical properties and spectral data: mp 293–294 °C; ESI-MS *m/z* 149.84 [$M^-$]; $^1$H NMR (600 MHz, MeOD) $\delta$ 6.90 (d), 6.70 (dd), 6.64 (dd); $^{13}$C NMR (151 MHz, EtOD) $\delta$ 155.39, 144.66, 141.66, 121.55, 117.54, 109.88, 100.22, 47.43.

### 2.3. Animals experiments

Male C57BL/6J mice (Quality certificate number: SCXK Xiang 2019-0004), weighing 20 ± 2 g, were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China), and housed at the well-prepared animal laboratory (22 ± 2 °C, 12 h light/dark cycle) with standard lab chow and water freely. All animal experiments were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Guangxi Medical University (Ethic Committee number: 201901053), China.

The animals were divided into six groups randomly: normal control group (n = 10 per group), model group, positive control group, and HBO-treated groups (high-, medium- and low-dosages). The normal control group and model group were pre-treated intragastrically with 0.5% CMC-Na. In addition, the HBO and Bifendate were prepared with 0.5% CMC-Na, and the positive control group was pre-treated intragastrically with Bifendate (150 mg/kg/d), and the HBO-treated groups were pre-treated intragastrically with HBO (200, 100 and

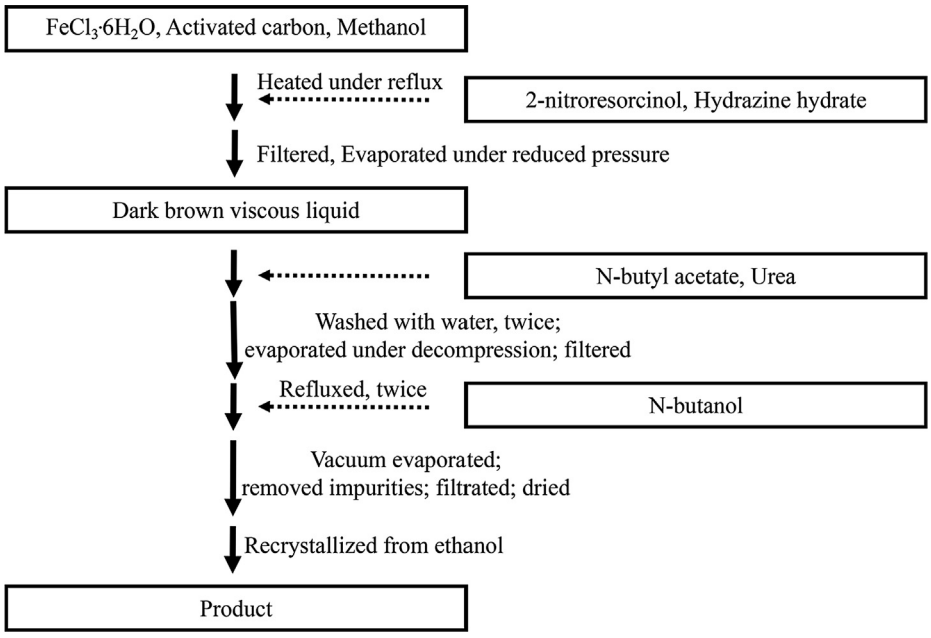


**Fig. 1.** A schematic synthesizing route illustrating the preparation method of HBO.

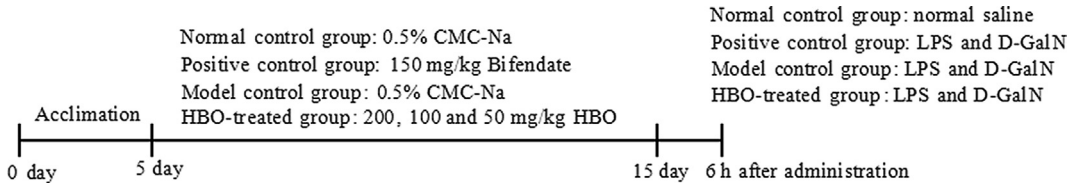


**Fig. 2.** The experiment schedule.

50 mg/kg/d). After 10 days, the normal control group was given one intraperitoneal injection of normal saline. The LPS and D-GalN were dissolved in normal saline, and all mice except the normal control group were given one intraperitoneal injection of LPS (10 μl/kg) and D-GalN (700 mg/kg). About 6 h later, all mice were sacrificed; moreover, the serum was collected and liver samples were separated immediately for further analysis (Fig. 2).

### 2.4. Histological analysis

A part of each liver sample was fixed in 4% paraformaldehyde for 48 h, embedded in paraffin and sliced into 5-μm sections. Then, the sections were stained by hematoxylin and eosin (H&E) and examined under a light microscope [10]. Moreover, the Terminal dUTP nick end labeling (TUNEL) method was used to detect hepatocyte apoptosis [11].

### 2.5. Preparation of tissue homogenate and mitochondria

#### 2.5.1. Preparation of tissue homogenate

Liver tissue samples were homogenized with cold Tris buffer (pH 7.4) on ice, and the homogenates were centrifuged at 4 °C. Then, the supernatants were divided and stored at −80 °C.

#### 2.5.2. Preparation of mitochondria

Mitochondria were prepared using a commercially-available kit. First, fresh liver tissue samples were washed with PBS, cut by scissors, and homogenized with Lysis Buffer. Next, the homogenate was centrifuged at 4 °C several times according to the instructions, and the extracted cytoplasm was preserved. Moreover, the pellet was re-suspended in the Wash Buffer and the mixture was centrifuged. At last, the final pellet was resuspended in the Store Buffer.

### 2.6. Evaluation of liver function

The levels of ALT, AST and TBIL were determined using commercially-available kits to assess the severity of the liver injury.

### 2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines in tissue homogenates such as TNF-α and IL-1β were examined using ELISA kits in accordance with the kit instructions.

### 2.8. Determination of oxidative stress markers

The activities of NO, iNOS, MDA, GSH-PX, GSH, SOD and CAT in liver homogenates were measured by assay kits, and the protein concentrations in liver homogenates were tested by the BCA protein detection kit. All assays were performed according to the manufacturer's instructions.

### 2.9. Immunohistochemistry

The protein expression of c-jun in livers was detected by immunohistochemical analysis method. Briefly, liver samples were sectioned into slices as described previously [10]. The sample slices were incubated with the primary antibody against c-jun (1:500, Proteintech Group, Inc., Wuhan, China) at 4 °C overnight. After washing, a species-specific secondary antibody was incubated and applied for 60 min at room temperature. Lastly, the samples were stained by horseradish peroxidase and diaminobenzidine; moreover, samples were re-stained by hematoxylin [12].

### 2.10. Real-time PCR (RT-PCR) analysis

PCR experiment was conducted with reagent kits (Takara Biomedical Technology, Beijing, China) following the manufacturer's protocol. The mRNA levels of TNF-α, IL-1β, TLR4, NF-κB p65, NF-κB p50, ERK, p38, JNK and c-jun were tested. Firstly, total RNA from liver samples of mice was isolated by the Trizol method. Secondly, cDNA was synthesized by removing genomic DNA reaction and reverse transcription reaction. Finally, the PCR reaction solution was prepared and then the real-time PCR reaction was performed with an Applied Biosystems Prism 7300 Fast Sequence Detection System, and the experience was performed with the following conditions: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 31 s at 60 °C. Moreover, GAPDH was used as an internal control. The result of each gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method according to the formula $RQ = 2^{-\triangle\triangle Ct}$ [13]. The primers used in the studies were listed in Table 1.

### 2.11. Western blot assay

The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis, then subsequently

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

| Gene | Forward primers (5′–3′) | Reverse primers (5′–3′) |
|---|---|---|
| TNF-α | GCCAGGAGGGAGAACAGAAACTC | GGCCAGTGAGTGAAAGGGACA |
| IL-1β | TCCAGGATGAGGACATGAGCAC | GAACGTCACACACCAGCAGGTTA |
| TLR4 | TCCTGTGGACAAGGTCAGCAAC | TTACACTCAGACTCGGCACTTAGCA |
| NF-κB p65 | ATTGCTGTGCCTACCCGAAAC | TTTGAGATCTGCCCTGATGGTAA |
| NF-κB p50 | TCCGGGAGCCTCTAGTGAGAA | TCCATTTGTGACCAACTGAACGA |
| ERK | TCCTGCTGAACACCACTTGTGA | CCAACGTGTGGCTACGTACTCTG |
| p38 | GATGAGCCTGTTGCTGACCCTTA | TGGTGGCACAAAGCTGATGAC |
| JNK | GACACTTGACAGGATAGGATGAGCA | CTAGCGCTGAGGCAACACTGA |
| c-jun | ATCCACGGCCAACATGCTC | ACGTTTGCAACTGCTGCGTTAG |
| GAPDH | TGTGTCCGTCGTGGATCTGA | TTGCTGTTGAAGTCGCAGGAG |

transferred to PVDF membranes (Millipore, USA). After blocking with 5% skim milk, the PVDF membranes were incubated at 4 °C overnight with the primary antibodies: TLR4 (1:500, Abcam), MyD88 (1:1000, Abcam), p-IKK-α/β (1:1000, Cell Signaling Technology Inc), IκB-α (1:1000, Cell Signaling Technology Inc), p-IκB-α (1:1000, Cell Signaling Technology Inc), NF-κBp50 (1:2000, Abcam), NF-κBp65 (1:500, Abcam), p-NF-κBp65 (1:1000, Abcam), MEK1 (1:1000, Boster), p-MEK1 (1:1000, Boster), ERK1/2 (1:1000, Cell Signaling Technology Inc), p-ERK1/2 (1:1000, Cell Signaling Technology Inc), JNK1/2 (1:1000, Cell Signaling Technology Inc), p-JNK1/2 (1:1000, Cell Signaling Technology Inc), p38 (1:1000, Cell Signaling Technology Inc), p-p38 (1:1000, Cell Signaling Technology Inc), Bcl-2 (1:1000, Proteintech), Bcl-xl (1:1000, Proteintech), BAX (1:1000, Boster), BAK (1:1000, Boster), and GAPDH (1:2500, Abcam).

After that, the membranes were washed three times with TBST and were incubated with fluorescence-labeled rabbit anti-goat IgG (Licor, USA) at 1:10,000 dilution at room temperature for 1 h. Finally, the membranes were washed again; and the bands were observed by Image Studio software.

In addition, the expressions of Cytochrome C (Cyt C) (1:2500, Abcam) in the mitochondria and cytoplasm were examined using Western blotting, and the procedure was the same as previously described procedures.

### 2.12. Statistical analysis

Statistical analysis was conducted using SPSS 11.5 for Windows. Data from the different groups were subjected to the one-way analysis of variance (ANOVA) followed by Tukey's test for post-hoc comparisons. All data were presented as means ± SD. The difference was considered statistically significant if p-value $< 0.05$.

## 3. Results

### 3.1. HBO exerted a protective effect on LPS/D-GalN-induced acute liver injury

The livers in each group of mice were shown in Fig. 3A. The samples in the normal group were light red in color and nice smooth in appearance. Obviously, the livers in LPS/D-GalN-treated group were deep red in color, rough in appearance and swelling in size as compared with the normal control group, and the livers of HBO-treated groups and Bifendate-treated group had been improved as compared with the model group, particularly in the high HBO-treated group and Bifendate-treated group. To further assess the extent of the damage in the liver, this study also evaluated histological changes and biochemical markers (serum levels of AST, ALT and TBIL). As shown in Fig. 3A, H&E staining revealed that the livers in the normal control group exhibited the normal structure with hepatic cells in the ordered arrangement and no pathological lesion area. But LPS/D-GalN-treated group was found to have the architecture disruption, characterized by massive necrosis, hemorrhage and inflammatory infiltration. It should be noted that the damaged tissues induced by LPS/D-GalN were alleviated both in Bifendate and HBO treatment groups. Moreover, treatment with Bifendate and 200 mg/kg HBO evidently reduced the percentage of necrotic area. In addition, serum AST, ALT and TBIL levels were strongly elevated by LPS/D-GalN treatment, however, their activities were decreased by the pre-treatment of Bifendate and HBO (Fig. 3B and C). These data clearly indicated that HBO could alleviate the severity of LPS/D-GalN-induced acute liver injury.

### 3.2. HBO suppressed LPS/D-GalN-induced hepatic oxidative stress

The anti-oxidative stress effect of HBO was evaluated by determining the levels of GSH, GSH-PX, SOD, MDA and CAT in hepatic tissues. As shown in Fig. 4A–D, the levels of GSH, GSH-PX, SOD and CAT were significantly decreased in LPS/D-GalN-treated group as compared to the normal control group, interestingly, these alterations induced by LPS/D-GalN were reversed by HBO and Bifendate treatment. Moreover, the LPS/D-GalN treatment led to an increase in MDA content, which was partially alleviated by HBO and Bifendate treatment (Fig. 4E). These data indicated that the protective effect of HBO on liver injury might be associated with its ability to reduce oxidative stress.

### 3.3. HBO alleviated inflammatory response

The production of NO is related to iNOS, and the high expression of iNOS can lead to organ destruction in some inflammatory. The result showed that the content of NO and iNOS was significantly higher in the LPS/D-GalN-treated group compared with the normal group (Fig. 4F–G). In contrast, HBO and Bifendate treatment largely decreased the levels of NO and iNOS. Inflammatory cytokines play an important role in LPS/D-GalN-induced acute liver injury, especially TNF-α, therefore the levels of TNF-α and IL-1β were tested. As indicated in Fig. 4H, the content of TNF-α and IL-1β was strikingly increased after LPS/D-GalN treatment, whereas HBO and Bifendate pre-treatment significantly reduced the over-expressions of these inflammatory cytokines. In addition, this study also detected the levels of TNF-α and IL-1β mRNA by RT-PCR. Obviously, the TNF-α and IL-1β mRNA expression levels were increased after LPS/D-GalN treatment, while HBO and Bifendate could inhibit this elevation to a large extent (Fig. 4I). These data suggested that HBO was able to ameliorate LPS/D-GalN-induced acute liver injury by alleviating inflammatory response.

### 3.4. HBO mitigated LPS/D-GalN-induced hepatocyte apoptosis

In the present study, TUNEL staining was used for examining hepatocyte apoptosis. The TUNEL result was shown in Fig. 5A. Brownish black dots suggested that LPS/GalN induced massive hepatocyte apoptosis, whereas HBO and Bifendate treatment markedly decreased these effects. Moreover, the expression levels of apoptosis-related proteins were examined by Western blotting. As shown in Fig. 5B, LPS/D-GalN treatment significantly up-regulated the expression of pro-apoptotic protein BAX and BAK, which were decreased when treated with HBO and Bifendate; furthermore, the expression of anti-apoptotic protein Bcl-2 and Bcl-xl were contrary to the pro-apoptotic protein.

In order to further explain the apoptotic reaction of the hepatic cell, mitochondrial Cyt C and cytosolic Cyt C were detected. In the present work, the ratio of mitochondrial to cytosolic Cyt C in normal group was markedly higher than the model group, however, HBO and Bifendate pre-treatment effectively restored this ratio (Fig. 5C). The results demonstrated that HBO and Bifendate could suppress Cyt C release from mitochondria into the cytoplasm. These results indicated that HBO had a strong inhibitory effect on apoptosis.

### 3.5. Effects of HBO on the change of the TLR4/NF-κB signaling pathway in LPS/D-GalN-induced acute liver injury

The TLR4 signaling pathway is considered to be an important pathway in modulating TNF-α, hence the effect of HBO on the TLR4/NF-κB pathway activation in the liver was measured. The results showed that the protein expression levels of NF-κBp50, TLR4 and MyD88, and the phosphorylation of IKK-α/β, IκB and NF-κBp65 in the LPS/D-GalN-treated mice liver were distinctly elevated compared with the normal control mice (Fig. 6A–E). After treatment with HBO and Bifendate, the expressions of NF-κBp50, TLR4 and MyD88, and the phosphorylation of IKK-α/β, IκB and NF-κBp65 aforementioned were significantly decreased. Moreover, the mRNA levels of TLR4, NF-κBp65 and NF-κBp50 in liver tissues were further examined by RT PCR. As shown in Fig. 6F, compared to the normal group, the mRNA expressions of TLR4, NF-κBp65 and NF-κBp50 were remarkably enhanced by LPS/D-GalN, however, the mRNA expression levels of these genes were

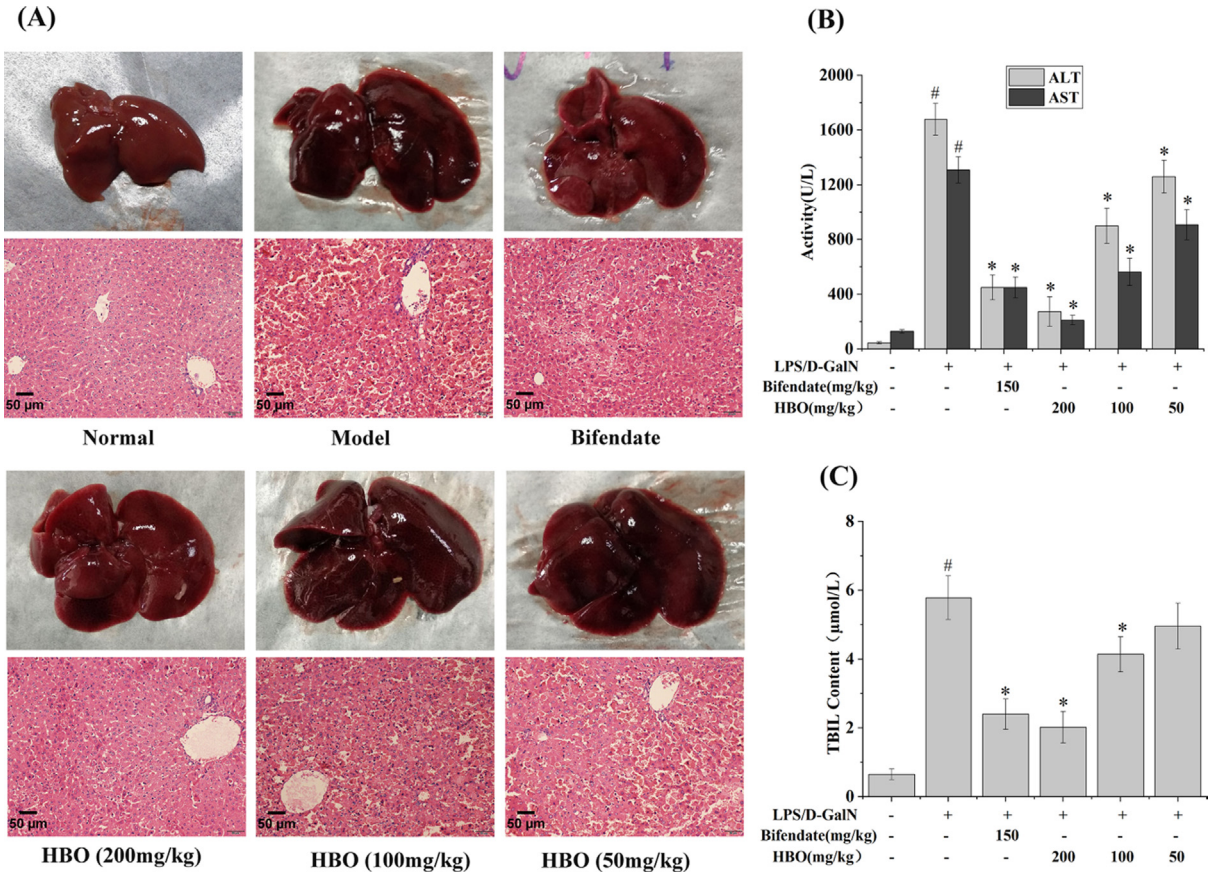


**Fig. 3.** HBO significantly alleviated liver injury in mice (n = 10). (A) Changes in liver morphology. And hepatic histological changes in each group were observed by H&E staining. (B to C) The activity of serum ALT, AST and TBIL were measured using an automatic biochemistry analyzer. Data are expressed as means ± SD. #P < 0.05 VS. the normal group; *P < 0.05 VS. the model group.

partially inhibited by HBO and Bifendate treatment. The above evidence demonstrated that HBO could alleviate the reversed abnormal expressions of the proteins in the TLR4/NF-κB pathway induced by LPS/D-GalN.

### *3.6. Effects of HBO on the change of the MAPK signaling pathway in LPS/D-GalN-induced acute liver injury*

Given the critical role of MAPKs family in LPS/D-GalN-induced acute liver injury, this study also examined the effect of HBO on the MAPK pathway in liver tissues. Fig. 7A–D showed that liver tissue levels of MEK, ERK1/2, JNK and p38 phosphorylations were significantly enhanced in LPS/D-GalN treatment mice compared to the normal group. Strikingly, the phosphorylation of these molecules was remarkably suppressed by HBO and Bifendate treatment. The protein level of c-jun in the liver was further examined by Immunohistochemical detection, and if there are brown granules in the nucleus, cytoplasm, cell membrane or interstitium, it will be positive cells. In our Immunohistochemical staining experiment, the positive signal of c-jun protein was localized in the cytoplasm and nucleus of liver injury tissue, showing brown-yellow granule shape. The liver section from the normal group revealed that c-jun was only faintly expressed; after challenged by LPS/D-GalN, the expression of c-jun was significantly observed, as the intense brown staining was shown in Fig. 7E. However, the expression of c-jun was markedly inhibited by treatment with HBO compared to the model group. To further explore whether HBO could inhibit the MAPK signaling pathway, RT-PCR analysis was carried out to determine the mRNA expression. As expected, with the treatments of LPS/D-GalN, ERK1/2, JNK, p38 and c-jun mRNA expressions were evidently enhanced. Nevertheless, the mRNA levels of ERK1/2, JNK, p38 and c-jun were remarkably reduced by treatment with HBO and Bifendate (Fig. 7F). These data indicated that the effects of LPS/D-GalN on the levels of ERK1/2, JNK and p38 phosphorylations were largely abolished by HBO.

## 4. Discussion

LPS/D-GalN-induced acute liver injury in mammals is a well-known model that has been widely used to simulate the formation of acute liver injury in humans and elucidate the exact pathogenesis [2]. It has been authenticated that LPS could lead to the macrophage activation and inflammatory responses, and LPS is considered as an important factor in causing hepatic injury. Moreover, D-GalN, a hepatotoxic agent, can consume uridine monophosphate in the liver, inhibiting the glycoprotein, nucleic acid and lipid synthesis, damaging the hepatocytes and causing liver injury. It is noteworthy that D-GalN can enhance the toxic effects of LPS. In this study, the liver injury model was induced by LPS/D-GalN in mice. The histopathological results showed that HBO clearly ameliorated the widespread cellular necrosis and damages of liver architecture. Generally, under normal conditions, ALT and AST mostly exist in liver cells, however, they are transferred into the serum when the liver is damaged, causing a prominent increase in their serum levels. At present, ALT and AST have been regarded as a symbol of the hepatocyte injury, whose activation levels can assess the severity of liver damage [14,15]. Moreover, the content of TBIL in serum is the index of the functional transport ability in the liver [16]. In the present study, we found that the levels of ALT, AST and TBIL were significantly increased after LPS/D-GalN treatment, indicating the acute liver injury; conversely, the ALT, AST and TBIL levels were significantly attenuated by Bifendate and HBO treatment. The above results indicated that LPS/

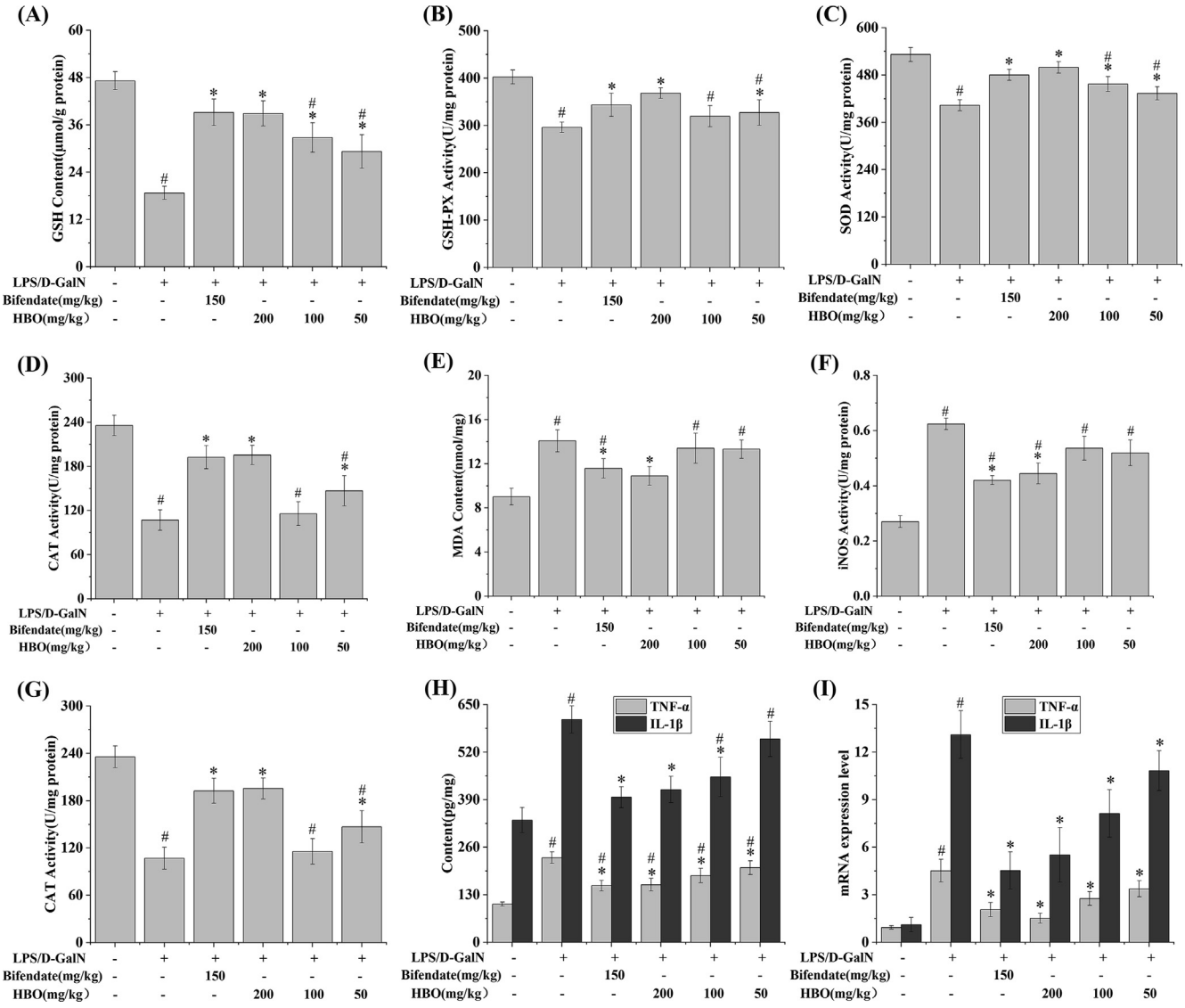


**Fig. 4.** HBO significantly alleviated lipid peroxidation and inflammatory response. (A to G) The levels of GSH, GSH-PX, SOD, CAT, MDA, iNOS and NO were detected using commercially available kits. (H) The content of TNF-α and IL-1β was determined by enzyme-linked immunosorbent assay. (I) The mRNA levels of TNF-α and IL-1β were determined using RT-PCR assays. Values are expressed as means ± SD (n = 10 in each group). #P < 0.05 VS. the normal group; *P < 0.05 VS. the model group.

D-GalN-induced acute liver injury was dramatically alleviated by HBO.

Oxidative stress plays a crucial role in the onset of LPS/D-GalN-induced acute liver injury. GSH is a major naturally-occurring antioxidant, and it can prevent oxidative damage by scavenging free radicals; moreover, GSH-Px, as a free radical scavenger, can catalyze the decomposition of hydrogen peroxide. Furthermore, SOD is an antioxidative enzyme, which plays a vital role in the balance of oxidation and anti-oxidation. And SOD can catalyze the disproportionation to produce hydrogen peroxide, while CAT accelerates the dismutation reaction of hydrogen peroxide. That is, the functions of SOD and CAT are interrelated, and both of them can scavenge oxygen radical species, and protect the integrity of cells [17,18]. MDA is the final product of lipid peroxidation, and the content of MDA can indicate the extent of oxidative damage in the liver [19]. In this study, after treatment with LPS/D-GalN, the levels of GSH, GSH-Px, SOD and CAT were significantly decreased, and the levels of MDA was evidently increased, demonstrating the serious oxidative injury in the liver. In contrast, HBO and Bifendate treatment could increase the levels of SOD, GSH, CAT and decrease the content of MDA. The results showed that the antioxidant capacity of HBO might be one of its important protective mechanisms against LPS/D-GalN-induced acute liver injury.

The inflammatory response is a major contributing factor in the development of acute liver injury induced by LPS/D-GalN. LPS excites kupffer cells, causing the excretion of various pro-inflammatory cytokines such as TNF-α and IL-1β [20]. Neutrophil activation and infiltration are elicited by TNF-α, thus TNF-α has been regarded as the main cellular regulator in the early inflammatory response, and it is a central mediator in the progress of LPS/D-GalN-induced acute liver injury [21]. In common with the TNF-α, IL-1β is a significant pro-inflammatory cytokine involving in liver injury, which could activate lymphocytes and lead to the release of other cytokines. Consequently, several pieces of evidence proved that regulation of the inflammatory response, especially the production of TNF-α, is an effective way for the treatment of the acute liver injury. In addition, NO could serve the function of regulating inflammatory response [22], and the level of iNOS could be an indicator of the degree of inflammation [23]. In the present study, the levels of TNF-α, IL-1β, NO and iNOS in the liver were evidently heightened after LPS/D-GalN treatment; similarly, the TNF-α and IL-1β mRNA levels were significantly increased. Interestingly, treatment with HBO and Bifendate noticeably reduced the contents of

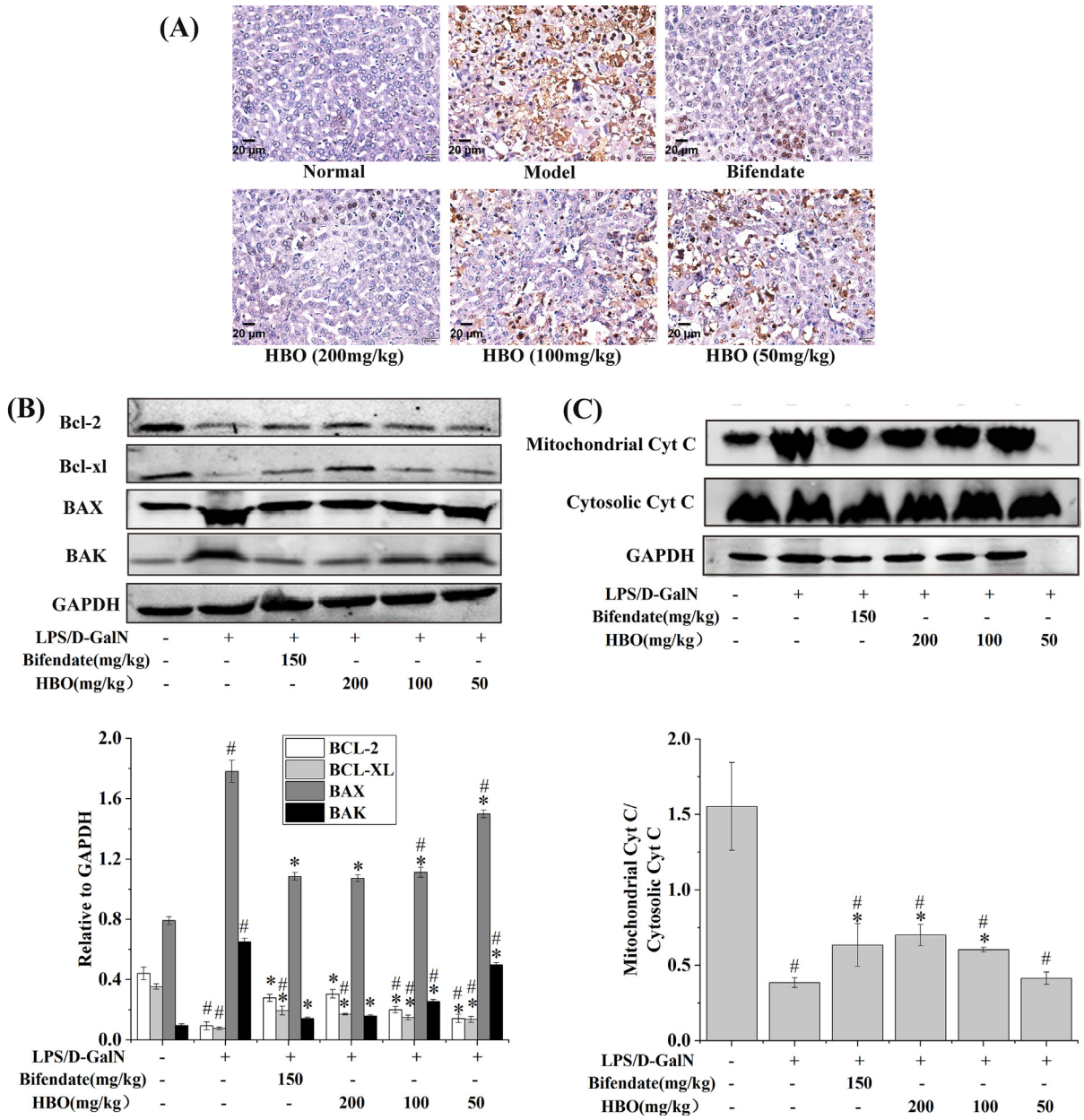


**Fig. 5.** Inhibition of LPS/D-GalN-induced hepatocyte apoptosis by HBO. (A) TUNEL staining was performed to observe apoptotic cells in liver tissue sections. (B to C) Apoptosis-related proteins levels were determined by Western blot. Data are presented as means ± SD (n = 3 in each group). #P < 0.05 VS. the normal group; *P < 0.05 VS. the model group.

TNF-α, IL-1β, NO and iNOS, as well as the mRNA levels of TNF-α and IL-1β, suggesting that the protective effects of HBO against liver injury induced by LPS/D-GalN probably through the restraint of inflammatory response.

In the LPS/D-GalN-induced acute liver injury model, the high percentage of hepatic cells apoptosis is a common phenomenon. Hence, hepatocyte apoptosis is a momentous target for ameliorating liver injury [24]. TUNEL staining can visually observe the cellular apoptosis of hepatic tissue. In addition, the expression of apoptosis-related proteins was being noted in this study. Apoptosis may occur by the mitochondrial pathway. The release of mitochondrial cytochrome c into the cytoplasm is a crucial event in controlling apoptosis progress. Furthermore, the Bcl-2 family is reported to be the key regulator for the maintenance of the membrane stability of mitochondria. The up-regulation of pro-apoptosis proteins such as BAX and BAK and down-regulation of anti-apoptosis proteins such as Bcl-2 and Bcl-xl change permeability of the mitochondrial membrane, which could lead to the overproduction and release of Cyt C by mitochondria [25]. In this study, the results showed that HBO significantly reduced hepatocyte apoptosis. Moreover, HBO pre-treatment effectively inhibited the expression of BAX, BAK, while increased the levels of Bcl-2 and Bcl-xl. HBO also reduced Cyt C release from mitochondria to cytosol. Hepatocyte apoptosis has been identified as an important pathological manifestation of LPS/D-GalN-induced acute liver injury, and our results suggested that HBO treatment reduced the apoptosis obviously.

Since the TLR4/NF-κB pathway played a crucial role in the acute liver injury process induced by LPS/D-GalN, in this study the protein levels of TLR4/NF-κB pathway were examined. For actually, the expression of TLR4 was substantially increased by LPS/D-GalN, and then the NF-κB signaling pathway was activated; afterwards, the inflammatory response was triggered [20,26]. Accordingly, much attention has been given to the NF-κB pathway. NF-κB, a nuclear transcription factor, acts a pivotal part in LPS/D-GalN-induced inflammation and apoptosis [27]. In the inactive state, the p65/p50 translocation is

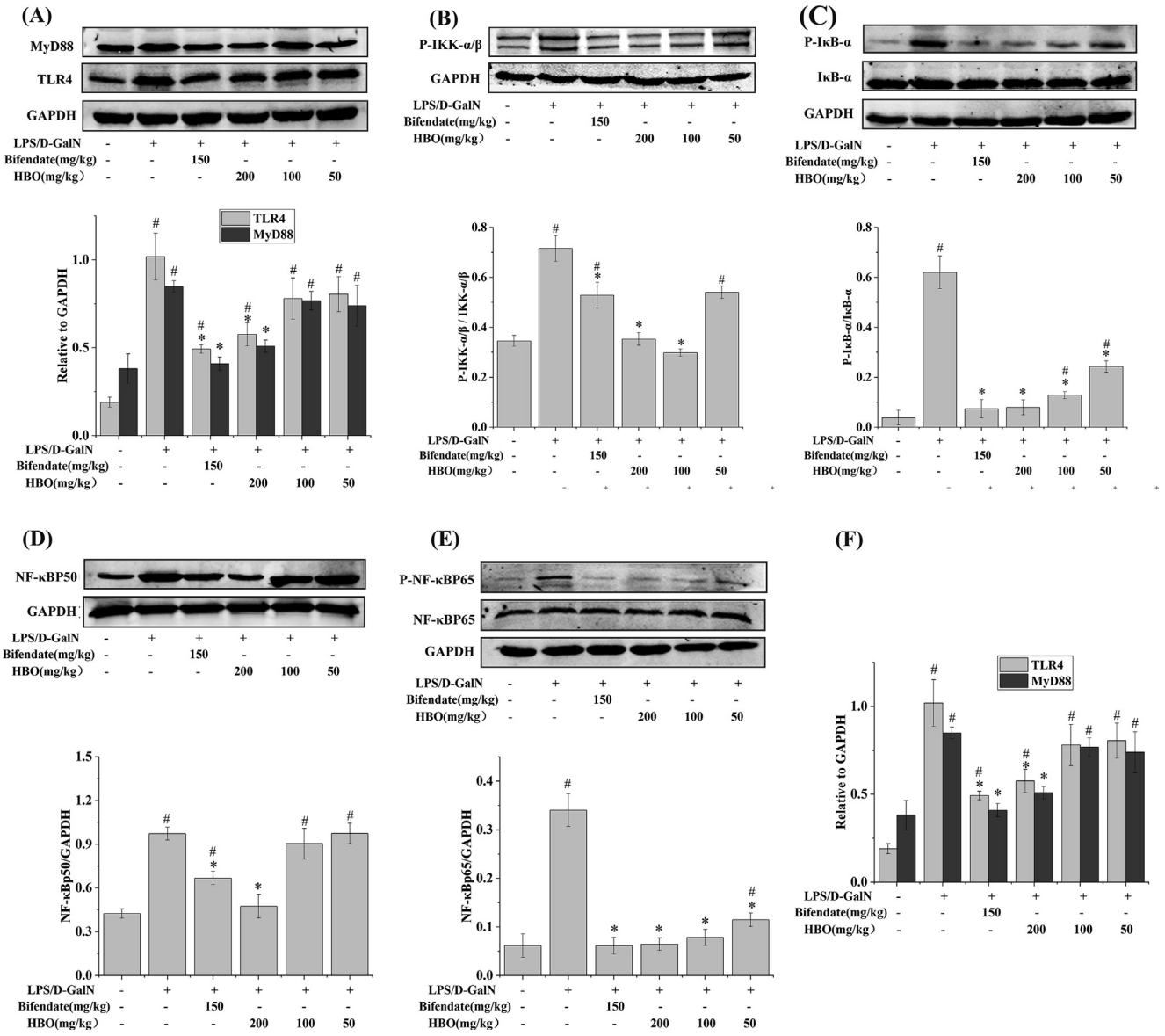


**Fig.6.** Effects of HBO on the change of the TLR4/NF-κB signaling pathway. (A–E) Protein levels were determined by Western blot. (F) The mRNA expression levels of TLR4, NF-κBP65 and NF-κBP50 were determined by RT-PCR assays. Data are presented as means ± SD (n = 3 in each group). #P < 0.05 VS. the normal group; *P < 0.05 VS. the model group.

commonly secluded in the cytoplasm owing to inhibitory protein IκBα. The IκB kinase (IKK) complex could be activated by various stimuli, next, the IκBα protein is phosphorylated. Then the p65/p50 heterodimer could transfer to the nucleus and activate the downstream gene transcription such as pro-inflammatory mediators [28,29]. Consistent with the previous studies, our results showed that TLR4 and NF-κB pathways were visibly activated by LPS/D-GalN [28]. Contrarily, HBO and Bifendate successfully reduced the expressions of TLR4, MyD88 and NF-κBp50, and markedly inhibited the phosphorylations of IKK-α/β, IκB-α and NF-κBp65. Similarly, HBO and Bifendate suppressed mRNA levels of TLR4, NF-κBp50 and NF-κB p65. The NF-κB pathway could be activated by TLR4, this is a recognized phenomenon in LPS/D-GalN-induced acute liver injury. And our results suggested that HBO could weaken the TLR4 expression and the phosphorylation of proteins in the NF-κB pathway, these findings indicated that HBO played a positive role in preventing TLR4/NF-κB activation.

MAPK signaling pathway also plays a key role in LPS/D-GalN-induced acute liver injury, accordingly, the MAPK signaling pathway was investigated. The MAPKs family can be stimulated by the activation of TLR4, and the MAPK signaling pathway appears to perform a considerable regulatory function of inflammatory response via its three major proteins including ERK, p38 and JNK. Moreover, a previous study has demonstrated that activation of the MAPK signaling pathway contributes to the production of multiple inflammatory factors (for example, TNF-α) [30]. ERK is involved in the regulation of cell proliferation, differentiation and survival. p38 mediates pro-inflammatory mediators and apoptosis, thus becomes a potential target for the anti-inflammatory cure. JNK plays a vital role in controlling apoptosis [31]. Indeed, we found that ERK, p38 and JNK pathways were activated by LPS/D-GalN, which is consistent with the previous studies [32]. Conversely, our data indicated that HBO and Bifendate treatment significantly diminished the phosphorylation of MEK, ERK, JNK, and p38, and inhibited the expression of c-jun. Additionally, the mRNA levels of JNK, ERK, p38 and c-jun were inhibited by HBO and Bifendate. These analyses indicated that HBO clearly repaired the enhanced of the MAPK signaling pathway.

In conclusion, our study demonstrates that HBO can ameliorate LPS/D-GalN-induced acute liver injury in mice. Surprisingly, HBO

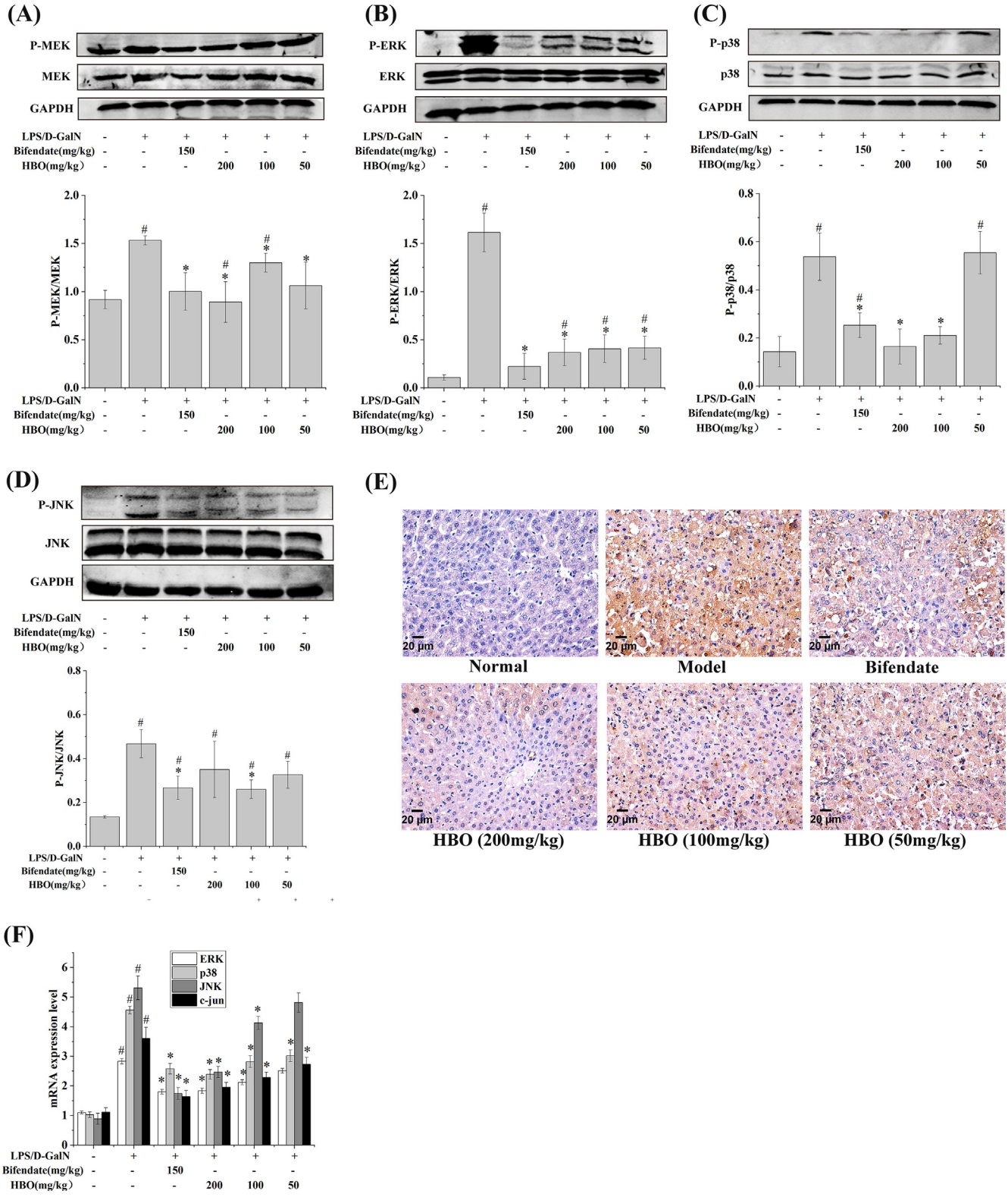


**Fig. 7.** Effects of HBO on the change of the MAPK signaling pathway. (A–D) Protein levels were determined by Western blot. (E) The expression of c-jun in liver tissues was analyzed by immunohistochemistry staining. (F) The mRNA expression levels of ERK, P38, JNK and c-jun were determined by RT-PCR assays. Data are presented as means ± SD (n = 3 in each group). #P < 0.05 VS. the normal group; *P < 0.05 VS. the model group.

treatment can down-regulate the phosphorylation of essential cytokines in TLR4/NF-κB and MAPK signaling pathways, and inhibit the further development of inflammation and hepatocyte apoptosis (Fig. 8). Our study probably provides a new insight for HBO as a potential agent for the treatment of liver injury.

## CRediT authorship contribution statement

**Hongyuan Wang:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft. **Xiugui Wei:** Formal analysis, Investigation, Data curation. **Xian Wei:** Methodology,

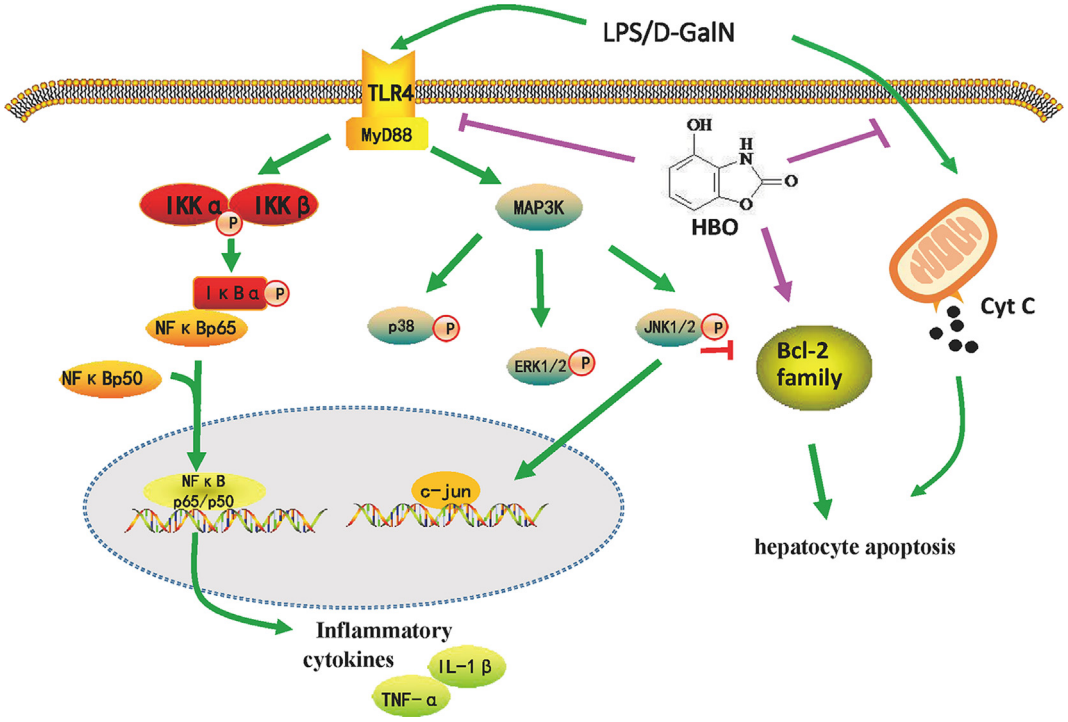


**Fig. 8.** A schematic diagram illustrating HBO ameliorates LPS/D-GalN-induced acute liver injury. (1) HBO regulated the expression of Bcl-2 family, and protected mitochondria from damage induced by LPS/D-GalN, reducing hepatocyte apoptosis; (2) HBO down-regulated the phosphorylation of essential cytokines in the TLR4/NF-κB and MAPK pathways and consequently reduced inflammatory responses, alleviating hepatic injury.

Validation. **Xuemei Sun:** Methodology, Validation. **Xiukun Huang:** Methodology, Validation. **Yingqin Liang:** Validation, Investigation. **Wanpeng Xu:** Validation. **Xunshuai Zhu:** Resources. **Xing Lin:** Conceptualization, Writing - review & editing, Supervision, Project administration. **Jun Lin:** Conceptualization, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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