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ScienceDirect
Chinese Journal of Natural Medicines 2020, **18**(3): 186–195
doi: 10.1016/S1875-5364(20)30020-0
Chinese Journal of Natural Medicines

•Special topic•

Berbamine ameliorates ethanol-induced liver injury by inhibition of hepatic inflammation in mice

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Available online 20 Mar., 2020

[ABSTRACT] Alcoholic liver disease (ALD) has become one of the leading causes of death in the world. Berbamine (BM), a natural product mainly derived from *Berberis vulgaris L*, possesses multiple bioactivities as a traditional medicine. However, the protective effect of BM on ALD remains unknown. In this study, we investigated the effect of BM on ethanol-induced hepatic injury in mice and its underlying mechanism. It was shown that BM at 0.3125−40 μmol·L^{-1} had no effect on macrophages and hepatocytes proliferation. BM at 5−20 μmol·L^{-1} significantly inhibited lipopolysaccharide (LPS) or acetate-induced IL-1β and IL-6 mRNA expression in RAW264.7 cells. Moreover, BM treatment significantly inhibited LPS-induced p65 and STAT3 phosphorylation in RAW264.7 cells. Hepatic histopathology analysis showed that inflammatory cells infiltration and lipid accumulation were suppressed by 25 and 50 mg·kg^{-1} BM administration in ethanol-induced hepatic injury mouse model. Meanwhile, BM treatment significantly inhibited serum ALT and AST levels in ethanol-fed mice. Oil red O staining results showed that BM administration ameliorated hepatic lipid accumulation in ethanol-fed mice. Preventions of ethanol-induced hepatic injury by BM were reflected by markedly decreased serum and hepatic triglyceride (TG) and total cholesterol (TC) contents. Real-time PCR results showed that BM treatment significantly inhibited pro-inflammatory cytokines mRNA expression in ethanol-fed mouse liver. Remarkably, the mechanism of action of BM was related to the reduction of ethanol-induced NF-κB and STAT3 phosphorylation levels in liver. In addition, BM treatment significantly inhibited ERK phosphorylation but not JNK and p38 of MAPK pathway. Taken together, our results demonstrate a beneficial effect of BM on ethanol-induced liver injury *via* a mechanism associated with inactivation of NF-κB, STAT3 and ERK pathway, which gives insight into the further evaluation of the therapeutic potential of BM for ALD.

[KEY WORDS] Berbamine; Ethanol; Hepatic injury; NF-κB; Inflammation
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2020)03-0186-10

Introduction

Alcoholic liver disease (ALD) is one of the leading causes of morbidity and mortality in the world [1]. Combined with the binging drinking, excessive chronic alcohol consumption is closely associated with development of ALD, which ranges from alcoholic fatty liver to alcoholic hepatitis, alcoholic cirrhosis and hepatocellular carcinoma [2]. Hepatic inflammation and injury aggravation are hallmarks in ALD patients [3]. However, pathogenic mechanisms of ethanol-induced hepatic injury remain unclear. It is urgent to develop the novel potential therapeutic drug for ALD.

Hepatic inflammation triggered by macrophages and neutrophils plays a key role in the development of ALD. Long-term ethanol exposure alters the microbiota and permeability of gut, resulting in elevated release of lipopolysaccharide (LPS) to the portal blood, which in turn enters the liver and activates Toll-like receptors (TLRs) [4−5]. Pro-inflammatory cytokines and chemkines released from Kuppfer cells, neutrophils, infiltrating bone-marrow and peritoneal monocytes activate myofibroblasts and induce hepatocyte apoptosis, all of which contribute to liver damage and disease progression [6].

The increasing studies have shown that natural com-

[Received on] 28-Nov.-2019
[Research funding] This work was supported by the National Natural Science Foundation of China (NSFC) (No. 81701573), Key Technologies of Prevention and Control for Major and Infectious Diseases Project of Suzhou (No. GWZX201604) and Youth Medical Talent Project of Jiangsu (No. QNRC2016214).
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These authors have no conflict of interest to declare.

pounds from microorganisms, plants and marine organisms provide a vast source of pharmaceutical materials. Up to date, a lot of drugs for the treatment of ALD were initially identified from natural compounds [7−8]. Berbamine (BM), a natural product mainly derived from *Berberis vulgaris L*, possesses multiple bioactivities as a traditional medicine [9]. The previous study has reported that BM as a novel nuclear factor kappa B (NF-κB) inhibitor increases A20 level, reduces IKKα phosphorylation and blocks p65 nuclear translocation [10]. It was also reported that BM as a novel immunomodulatory agent selectively down-regulated STAT4 and IFN-*γ* in experimental autoimmune encephalomyelitis [11]. However, the protective effect of BM on fatty liver disease especially ALD remains unknown. The aims of this study are to investigate the roles and mechanisms of BM in ALD. It was found that BM treatment ameliorated ethanol-induced hepatic injury in mice *via* the inhibition of inflammation reaction. This effect was mainly attributed to its inhibition of NF-*κ*B, STAT3 and ERK signaling activation.

Materials and Methods

MTT assay

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD) and human normal hepatocyte cell line HL-7702 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium media supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin at 37 °C in 5% CO_2. Cells were treated with various concentrations of BM for indicated time, MTT assay was performed to measure cell viability by a plate reader (Molecular Devices, San Jose, CA).

Animals

Female C57BL/6J mice, 10−12 weeks of age, were purchased from the Laboratory Animal Center of Harbin Medical University (Harbin, China) and maintained on a standard diet and water ad libitum with a 12 h light/dark cycle. The animal experiment was carried out in accordance with the Guide for Care and Use of Laboratory Animals of Harbin Medical University. The experimental protocol used in this study was approved by the committee for the use of experimental animals of Harbin Medical University.

Ethanol feeding and treatment

An ethanol feeding mouse model was used as previous study [12]. C57BL/6J mice were administered a Lieber-DeCarli control liquid diet (#F1259SP, BioServ, Flemington, NJ) for 5 days. On the 6th day, mice were administered a Lieber-DeCarli liquid diet (#F1258SP, BioServ, Flemington, NJ) containing 5% (*V/V*) ethanol or pair-fed a Lieber-DeCarli control liquid diet for 10 days. On the 16th day, mice were gavaged with a single dose of ethanol (5 $g \cdot kg^{-1}$) or isocaloric maltose dextrin (9 $g \cdot kg^{-1}$). Mice were randomly divided into four groups (n = 6−10 per group). Ethanol-fed mice were injected with BM (25 or 50 $mg \cdot kg^{-1}$, APExBIO, Houston, TX) as previous study [11] or vehicle intraperitoneally once daily from day 6 to day 16. After a binge ethanol feeding for 9 h on the 16th day, liver tissues and serum were collected for further analysis.

Histology

Liver tissues from mice were fixed in 10% formalin and embedded in paraffin. Slices (5 μm) of liver tissues were deparaffinized in xylene and stained with hematoxylin and eosin (H&E) to evaluate the pathologic structures of the tissues. The clinical scores were determined as previous study [13] (0 = occasional inflammatory cells infiltration; 1= mild inflammation; 2 = severe inflammation; 3 = inflammation with fibrosis). Frozen liver tissues were embedded in OCT and Oil Red O staining was performed according to the manufacturer's instructions.

Determination of serum and hepatic lipid levels

The triglyceride (TG) and total cholesterol (TC) levels in serum and liver were analyzed using commercial kits according to the manufacturer 's instructions (Nanjing Jiancheng bioengineering institute, Nanjing, China). Liver samples (approximately 100 mg) were homogenized in a lysis solution using an electric homogenizer. Results of TG and TC levels in the serum were expressed as mmol per liter ($mmol \cdot L^{-1}$) and results of TG and TC levels in the liver were expressed as mmol per g protein ($mmol \cdot g^{-1}$protein).

Measurement of plasma ALT and AST

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) were quantified in serum using a commercial kit (Nanjing Jiancheng bioengineering institute, Nanjing, China).

Quantitative real-time PCR

Reverse transcription reactions and real-time PCR were performed as described previously [13]. Briefly, total RNA was isolated using Trizol reagent and cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer 's instructions. Real-time PCR was performed using GoTaq qPCR master mix (#A6001, Promega, Madison, WI) and Roche LightCycler detection system (Roche, Mannheim, Germany). Amplification of 36B4 was used as an internal control. Relative mRNA expression was quantified using the comparative CT method and expressed as $2^{(-\Delta\Delta^{Ct})}$. The sequences of the primers used for PCR amplification are listed in Table 1.

Western blot

Liver tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails (Cell Signaling Technology, Danvers, MA). The protein contents were determined by a BCA™ protein assay kit (Pierce, Rockford, IL). The protein lysates were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with primary antibody overnight at 4 °C and conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using a western blotting detec-

Table 1 The sequences of the primers used for PCR amplification

Gene		Primer sequence
TNF-α	sense	CTTCTGTCTACTGAACTTCGGG
	antisense	CAGGCTTGTCACTCGAATTTTG
IL-6	sense	CAAAGCCAGAGTCCTTCAGAG
	antisense	GTCCTTAGCCACTCCTTCTG
IL-1β	sense	ACGGACCCCAAAAGATGAAG
	antisense	TTCTCCACAGCCACAATGAG
Arg-1	sense	AAGAATGGAAGAGTCAGTGTGG
	antisense	GGGAGTGTTGATGTCAGTGTG
MCP-1	sense	GTCCCTGTCATGCTTCTGG
	antisense	GCTCTCCAGCCTACTCATTG
IL-10	sense	AGCCGGGAAGACAATAACTG
	antisense	GGAGTCGGTTAGCAGTATGTTG
36B4	sense	CACTGGTCTAGGACCCGAGAAG
	antisense	GGTGCCTCTGGAGATTTTCG

tion system according to the manufacturer's instructions. The densities of protein bands were analyzed by Image J software. GAPDH was used as an internal control. MAPK family antibody sampler kit (#9926), Phospho-MAPK family antibody sampler kit (#9910), anti-p-p65 (#3033), anti-p65 (#8242), anti-STAT3 (#9139), anti-p-STAT3 (#9145) and anti-GAPDH (#5174) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-SIRT1 (13161-1-AP) antibody was purchased from Proteintech (Chicago, IL).

Statistical analysis

All experimental data are presented as mean ± standard error of mean (SEM). The results were analyzed using one-way ANOVA followed by a Dunnett's test (GraphPad Prism 7.0 software). $P < 0.05$ was considered statistically significant.

Results

Berbamine inhibited LPS or acetate-induced inflammation in RAW264.7 cells

The structure of BM is presented in Fig. 1A. To determine the anti-inflammatory activity of BM, RAW264.7 and HL-7702 cells were treated with various concentrations of BM and MTT assay was used to evaluate the effect of BM on the cell viability. As shown in Fig. 1B, C and D, BM with 80 μmol·L^{-1} significantly inhibited cell proliferation. However, BM with the low concentrations has no effect on cell proliferation. Therefore, BM with the low concentrations (5−20 μmol·L^{-1}) were used for the following anti-inflammatory effect. Furthermore, the effect of BM on anti-inflammation was determined by real-time PCR. As shown in Figs. 2A and B, LPS and acetate significantly induced IL-1β and IL-6 mRNA

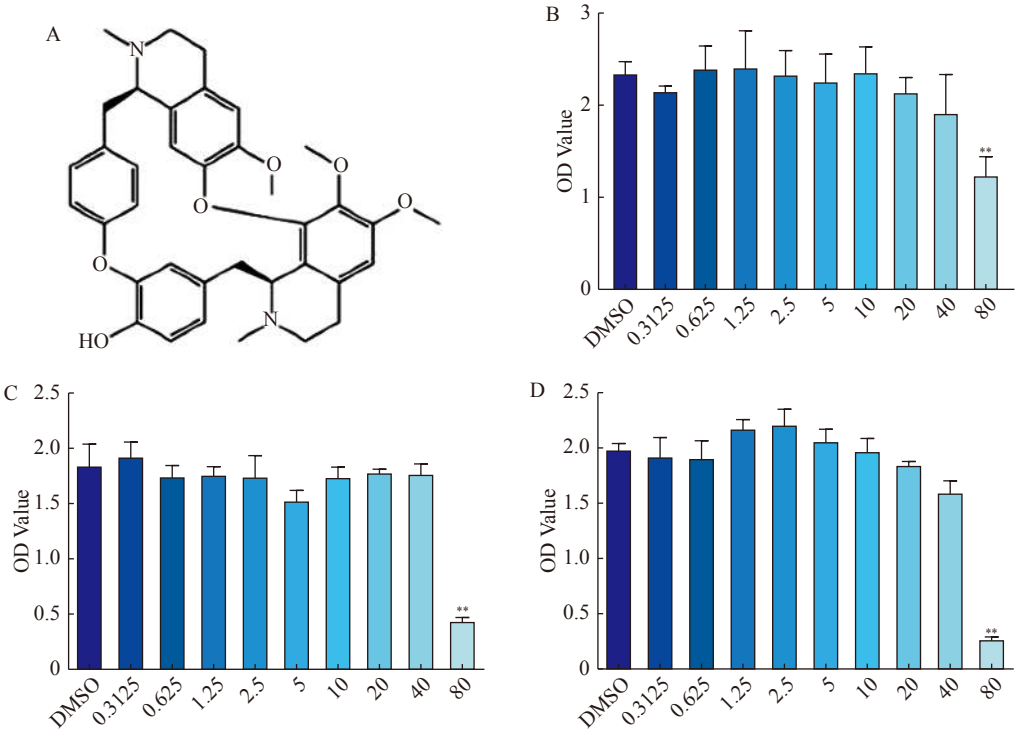

Fig. 1 The effects of Berbamine on cell proliferation. (A) The chemical structure of Berbamine (BM). (B) Raw264.7 cells were treated with indicated concentrations of BM for 6 h, the cell viability was determined by MTT assay. (C) HL-7702 cells were treated with indicated concentrations of BM for 6 h, the cell viability was determined by MTT assay. (D) HL-7702 cells were treated with indicated concentrations of BM for 24 h, the cell viability was determined by MTT assay. Results are shown as mean ± SEM. $^{**}P < 0.01$ *vs* control

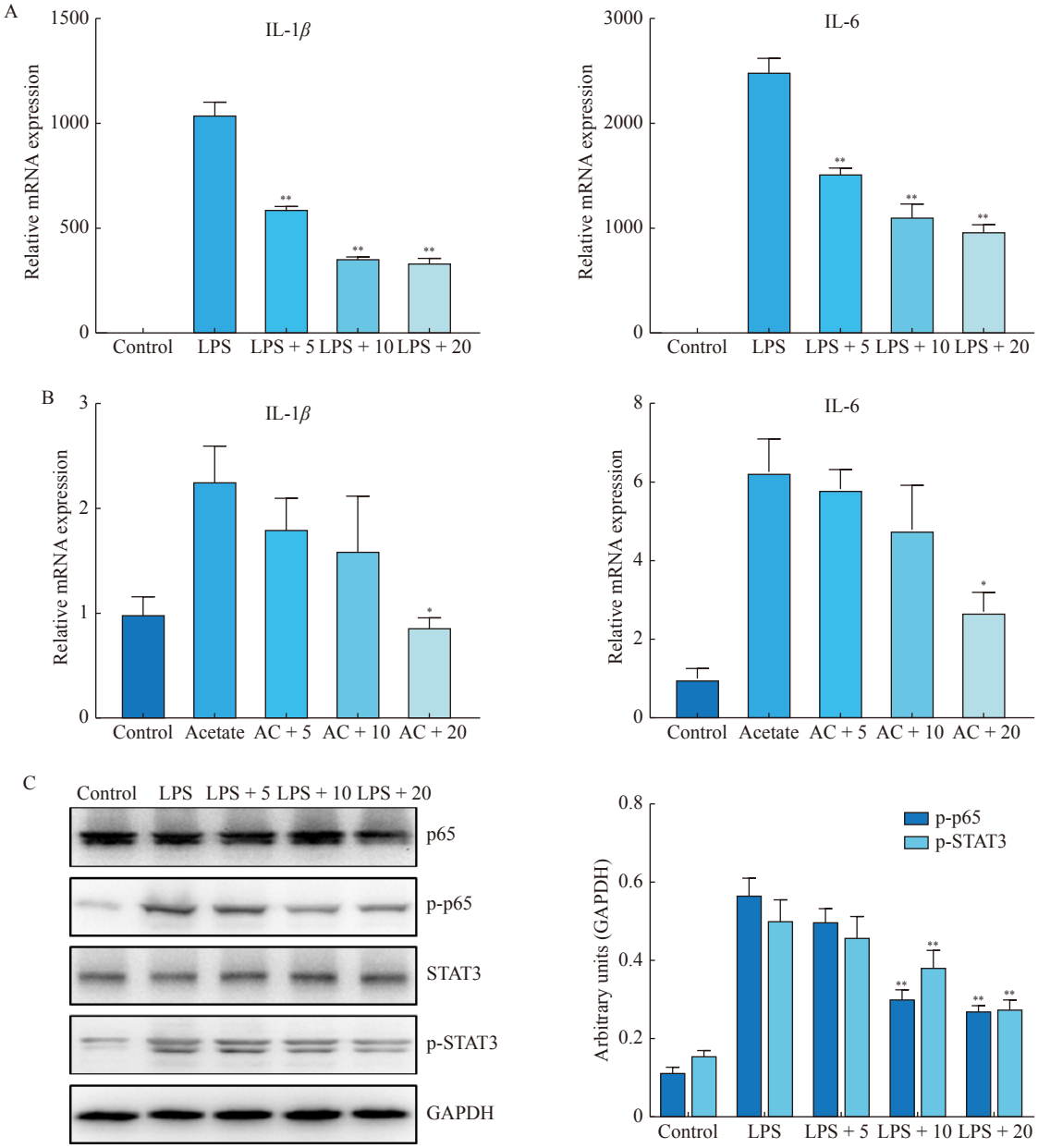

Fig. 2 Berbamine inhibited LPS or acetate-induced inflammatory response in Raw264.7 cells. (A−B) Cells were treated with indicated concentrations of BM for 6 h in the absence or presence of LPS (100 ng·mL^{-1}) or acetate (20 nmol·L^{-1}), the levels of IL-1*β* and IL-6 mRNA expression were determined by real-time PCR. (C) Cells were treated with indicated concentrations of BM for 30 min in the absence or presence of LPS (100 ng·mL^{-1}), the levels of p65 and STAT3 phosphorylation were determined by western blot analysis. The densities of protein bands were analyzed by Image J software. Results are shown as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control

expression in RAW264.7 cells. BM treatment inhibited LPS-induced IL-1*β* and IL-6 mRNA expression in a dose-dependent manner. Meanwhile, the treatment of BM at 20 μmol·L^{-1} significantly inhibited acetate-induced IL-1*β* and IL-6 mRNA expression. In addition, western blot showed that BM siginificantly inhibited LPS-induced p65 and STAT3 phosphorylation in RAW264.7 cells. These data indicated that BM might have anti-inflammatory effects.

Berbamine ameliorated ethanol-induced liver injury in mice

Mice administered vehicle, 25 or 50 mg·kg^{-1} BM were fed an ethanol diet for 10 days followed by a single gavage of ethanol. In control group, mice were fed a control diet for 10 days followed by a single gavage of maltose. As shown in Fig. 3A, histological analysis by H&E staining of the liver suggested ethanol feeding caused excessive inflammatory cells infiltration and lipid droplets accumulation. However, these effects were ameliorated by BM treatment. The infiltration of inflammatory cells into the liver was markedly reduced and few lipid droplets were found in BM-treated mice. Histopathological scores results showed that 25 and 50

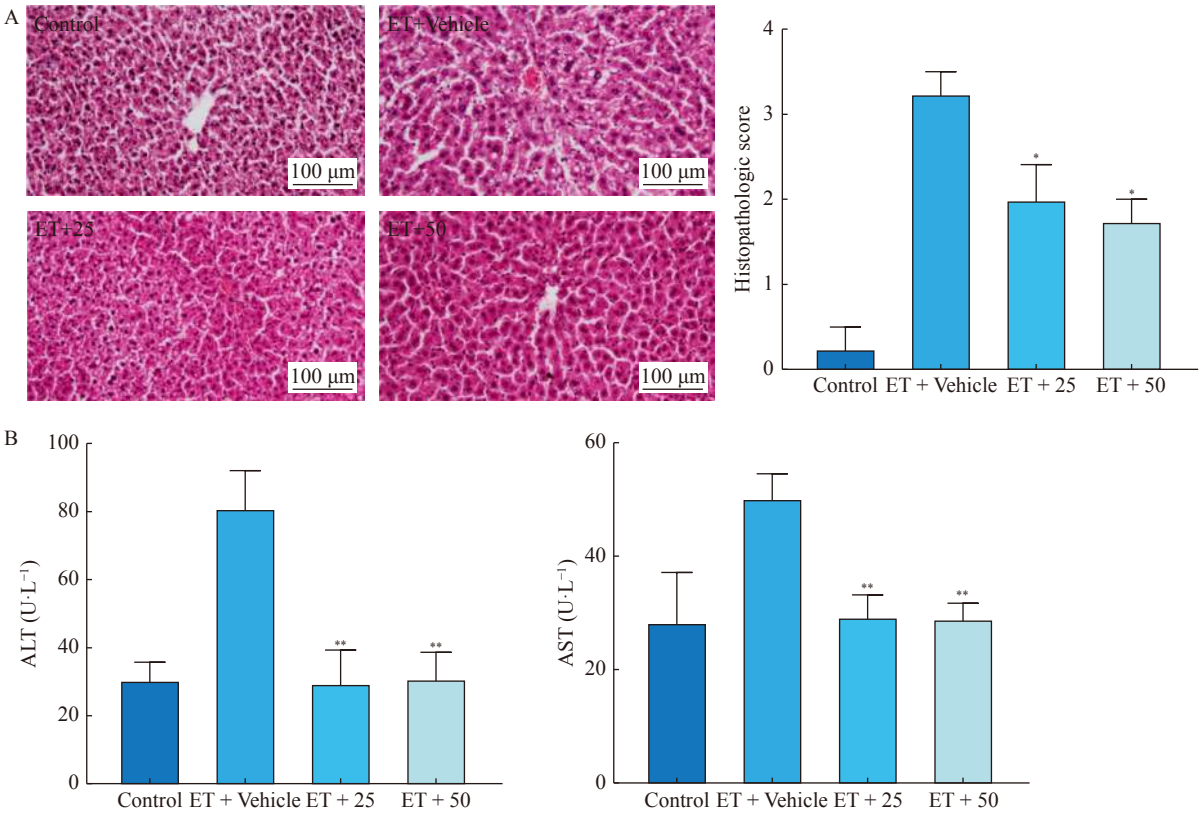

Fig. 3 Berbamine ameliorated ethanol-induced liver injury. Mice administered vehicle, 25 or 50 mg·kg^{-1} BM were fed an ethanol diet for 10 days followed by a single gavage of ethanol. In control group, mice were fed a control diet for 10 days followed by a single gavage of maltose. All mice were euthanized 9 h after gavage. (A) Representative hematoxylin and eosin staining from liver section (magnification: 200 ×). (B) Serum AST and ALT levels. Results are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* vehicle group ($n = 6–10$)

mg·kg^{-1} BM administrations significantly ameliorated ethanol-induced hepatic injury. In addition, serum ALT and AST levels, the markers of liver injury, markedly reduced by BM treatment in ethanol-fed mice.

Berbamine reduced lipid accumulation in ethanol-fed mice

Hallmarked by hepatic steatosis, ALD is associated with a multitude of detrimental effects. We used Oil red O staining to evaluate the effect of BM on lipid accumulation induced by ethanol feeding in mice. As shown in Fig. 4A, the ethanol feeding increased lipid accumulation compared with pair-fed control mice. BM treatment reduced lipid levels in liver. To confirm this effect of BM, serum and hepatic TG and total cholesterol (TC) levels were determined by commercial kits. Figs. 4B and C showed that BM treatment obviously reduced TG and TC levels in serum and liver, which contributed to amelioration of ethanol-induced liver injury.

Berbamine inhibited ethanol-induced pro-inflammatory cytokines mRNA expression in liver

Hepatic inflammation plays a key role in the development of ALD. RNA from liver was isolated and pro-inflammatory factors were determined by real-time PCR. As shown in Fig. 5, the ethanol feeding induced significantly pro-inflammatory mediators including IL-1β, IL-6, TNF-α and MCP-1 mRNA expression increasing. Interestingly, ethanol did not alter anti-inflammatory factors IL-10 and Arginase-1 (Arg-1) mRNA expression. BM treatment reduced these pro-inflammatory cytokines mRNA expression in liver. However, BM failed to alter IL-10 and Arg-1 mRNA expression in ethanol-fed mice. These results suggest that the reduction of hepatic inflammation by BM is responsible for the amelioration of ethanol-induced liver injury.

Berbamine reduced ethanol-induced NF-κB and STAT3 phosphorylation levels in liver

Inflammation is mainly regulated by transcription factors such as NF-κB and STAT3. To investigate the possible mechanisms, the activation of transcription factors NF-κB and STAT3 were determined by western blot. The results showed that ethanol increased NF-κB and STAT3 phosphorylation levels in liver, indicating that these two transcription factors activation increased. BM treatment significantly inhibited the phosphorylation levels of NF-κB and STAT3, which were consistent with the results of inflammatory factors mRNA expression decreasing (Fig. 6). In addition, many studies have been shown that SIRT1 exerts anti-inflammatory effects by deacetylation of modified lysine residues on transcription factor [14]. However, our data showed that BM did not have any effect on SIRT1 expression in ethanol-fed mice liver.

The effect of Berbamine on MAPK pathway in ethanol-fed mouse liver

To further investigate the signaling involved in the pro-

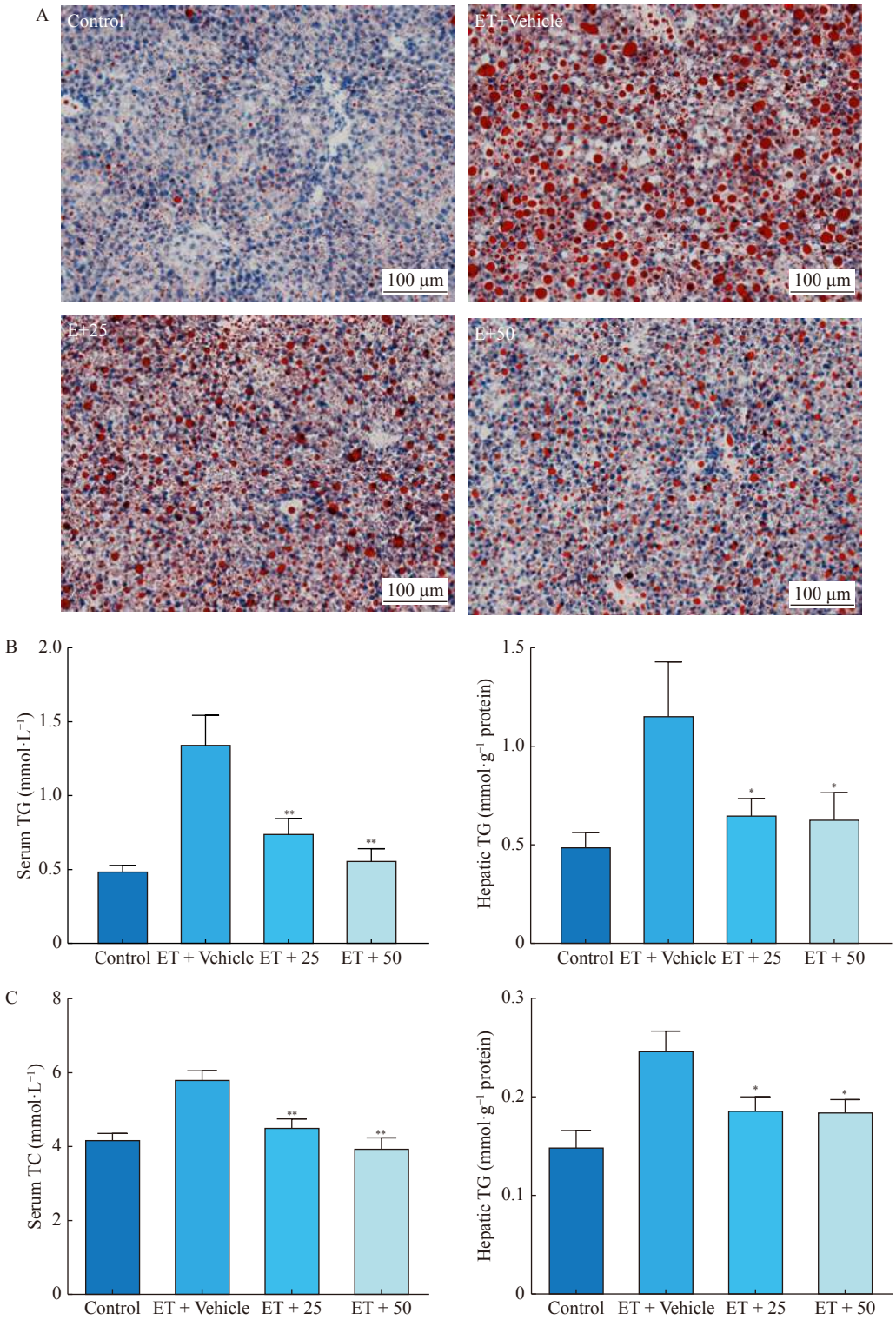

Fig. 4 Berbamine reduced ethanol-induced serum and hepatic lipid accumulation. Ethanol-fed mice were administered vehicle, 25 or 50 mg·kg^{-1} BM were fed an ethanol diet for 10 days followed by a single gavage of ethanol. (A) Representative Oil red O staining from liver section (magnification: 200 ×). (B) Serum and hepatic TG levels. (C) Serum and hepatic TC levels. Results are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* vehicle group (n = 6–10)

tective role of BM in ALD, the activation of MAPK pathway was determined by western blot. As shown in Fig. 7, compared to the vehicle-treated mice, BM at 50 mg·kg^{-1} significantly inhibited ERK phosphorylation levels in liver. However, the phosphorylation levels of JNK and p38 in MAPK pathway did not alter after BM treatment.

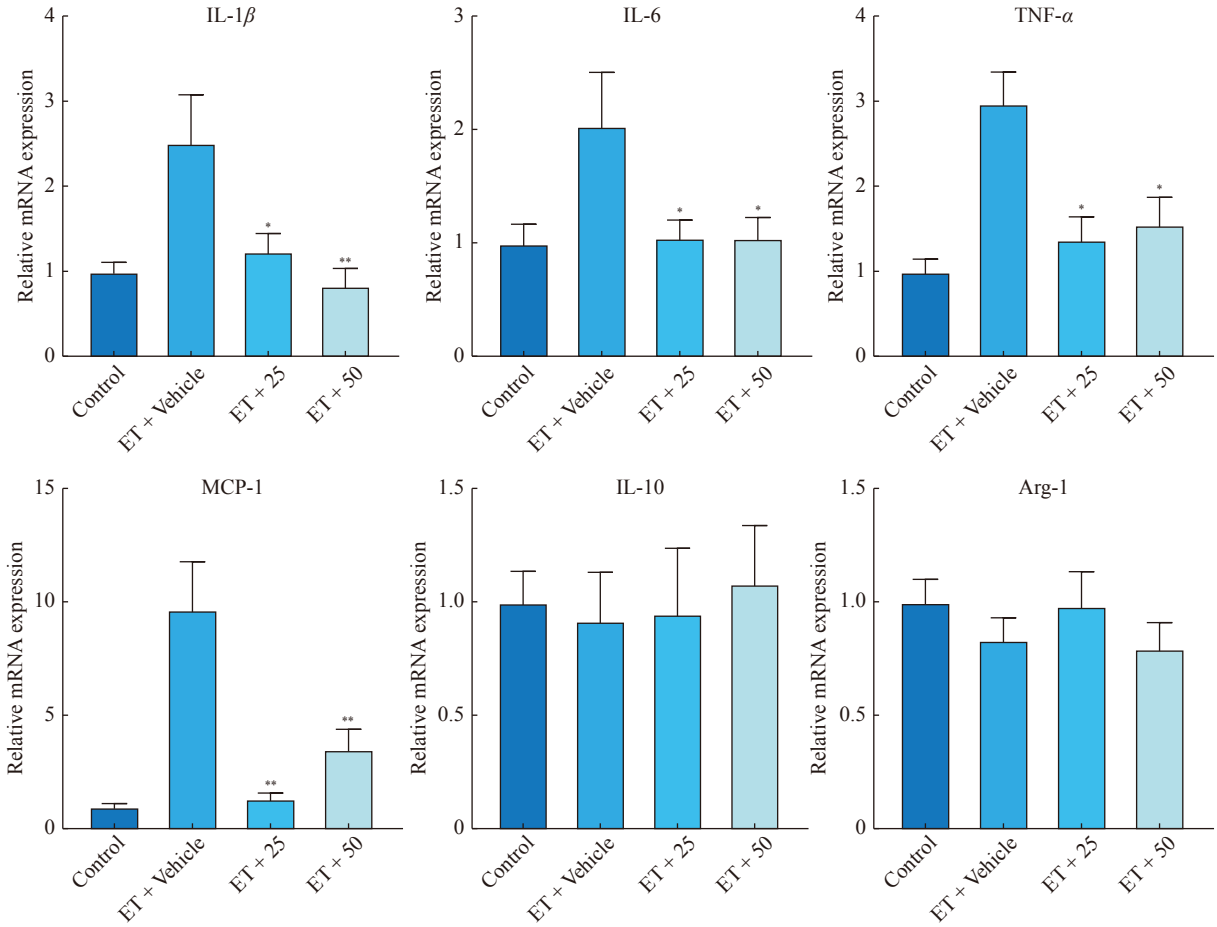

Fig. 5 Berbamine inhibited pro-inflammatory cytokine mRNA expression in ethanol-induced mouse liver. Ethanol-fed mice were administered vehicle, 25 or 50 mg·kg^{-1} BM were fed an ethanol diet for 10 days followed by a single gavage of ethanol. Total RNAs from liver tissues were isolated and mRNA levels were determined by real-time PCR. Results are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* vehicle group ($n = 6–10$)

Discussion

Berbamine isolated from *Berberis vulgaris L* is an important component of many traditional Chinese medicine. There were several reports about anti-cancer, anti-inflammatory activities of BM, which has been associated with numerous pharmacological effects [15]. Few studies tested the role of BM in liver disease even ALD. In this study, we aimed to investigate the possible therapeutic effect and underlying mechanism of BM on ALD. Our data revealed that treatment with BM could indeed ameliorate ethanol-induced liver injury in mice.

ALD is a serious health issue throughout the world whose incidences is on the rise with the past few decades. Excessive alcohol consumption is responsible for approximately 4% of all deaths annually and 5% of all disabilities worldwide [16]. Heavy chronic ethanol consumption produces a wide spectrum of hepatic lesions, which are characterized with hepatic steatosis, hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. Although abstinence has remained the primary therapeutic option for ALD treatment, serious symptoms develop with the abrupt cessation of alcohol and abstinence is difficult to maintain in most patients with ALD [17]. Therefore, to develop novel therapeutic interventions is the key in the treatment of ALD.

Numerous studies have been reported that hepatic inflammation plays a vital role in ALD [3]. The clinical trails reveal that corticosteroids and pentoxifylline are used in the treatment of alcoholic hepatitis by reducing pro-inflammatory cytokine production. However, these two agents have relatively strong side effects [6, 18]. Natural medicines have unique advantages in the treatment of various diseases with low toxicity and little side effect. Our data showed that BM has a low cytotoxicity to murine macrophages and strong anti-inflammatory effect including inhibiting LPS-induced IL-1β and IL-6 mRNA expression (Figs. 2A and B), indicating that BM might exist a protective role in the development of ALD. In this study, we used a NIAAA animal model [12] to investigate the effect of BM on ethanol-induced hepatic injury. Our data showed that BM ameliorates ethanol-induced liver injury and reduced serum and hepatic TG and TC contents (Figs. 3 and 4). Recently, the increasing studies have reported that BM has several protective activities. BM postconditioning confers ischemia/reperfusion-induced cardio

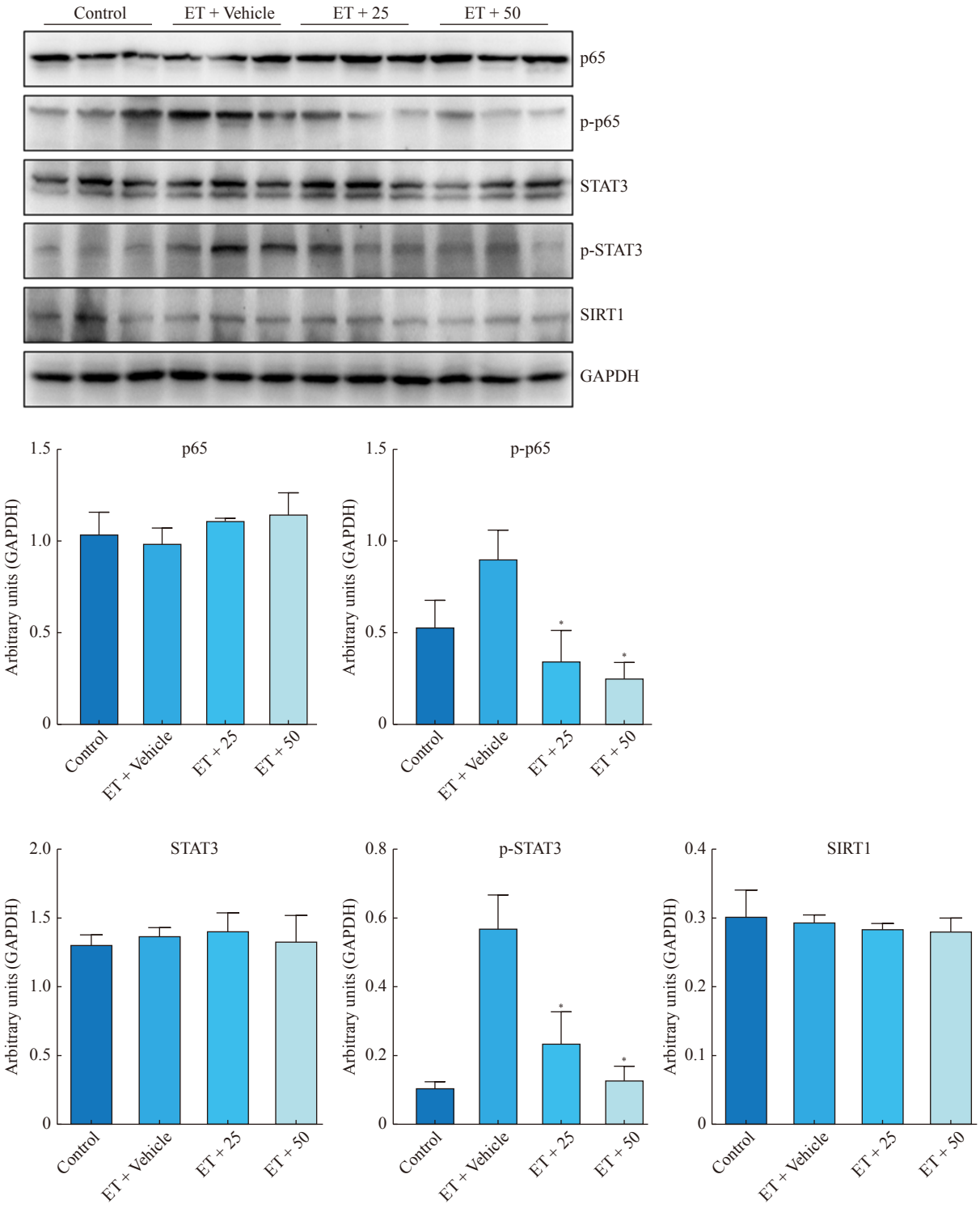

Fig. 6 Berbamine inhibited NF-κB and STAT3 activation in ethanol-fed mice. The expression of p65, phosphorylated p65, STAT3, phosphorylated STAT3 and SIRT1 from ethanol-fed mice liver were determined by western blot. GAPDH was used as an internal control. The densities of protein bands were analyzed by Image J software. Results are shown as mean ± SEM. $^{*}P < 0.05$ *vs* vehicle group

injury by modulation autophagy by activation of AKT pathway [19]. BM also ameliorates isoproterenol-induced myocardial infarction by inhibiting mitochondrial dysfunction and apoptosis in rats [20]. However, few studies reported the effect of BM on liver disease. The only study reported that BM ameliorated the activities of metabolic enzymes and maintained glucose homeostasis in high fat diet/streptozotocin-induced diabetic rats [21]. Our study first demonstrated that BM

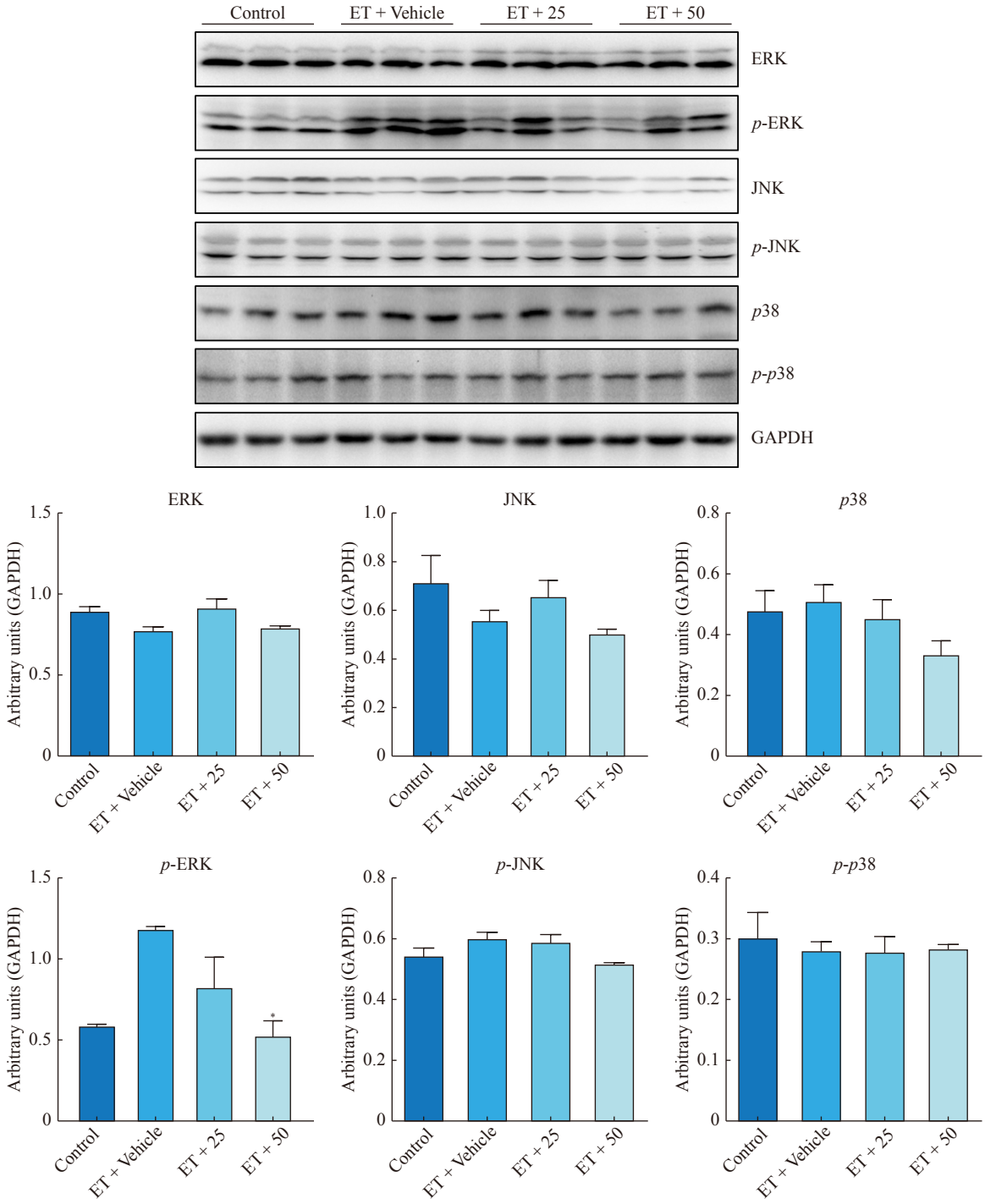

Fig. 7 The effect berbamine on MAPK pathway in ethanol-fed mice. The expression of ERK, phosphorylated ERK, JNK, phosphorylated JNK, p38 and phosphorylated p38 from ethanol-fed mice liver were determined by western blot. GAPDH was used as an internal control. The densities of protein bands were analyzed by Image J software. Results are shown as mean ± SEM $^{*}P < 0.05$ *vs* vehicle group

ameliorated ethanol-induced liver injury by inhibition of hepatic inflammation including suppression of pro-inflammatory cytokines production.

It is well known that inflammatory cytokine production was mediated by transcription factors including NF-κB and signal transducer and activator of transcription (STAT) [22–23]. Inflammatory cell infiltration is the hallmark of steatohepatitis in ALD. NF-κB, STAT and MAPK pathways are involved in the development of ALD [24]. Our study showed that BM inhibited the phorsphorylation level of NF-κB, STAT3 and ERK induced by ethanol-fed mice. Recently, it has been reported that BM has an anti-inflammatory potential, which is effected *via* inhibition of NF-κB and MAPK signaling pathways [25]. Many studies showed that BM inhibited cancer cell

proliferation *via* NF-*κ*B signaling [10, 26]. Although the effects of BM on the NF-*κ*B pathway have received some attention, we demonstrated here for the first time that BM exhibited an anti-inflammatory effect by NF-*κ*B, STAT and MAPK pathway *in vivo*, indicating that the inhibition of kinases could be a potential therapeutic option to ameliorate ALD by BM. However, it has been report that IL-6 improves ALD *via* the activation of STAT3 and the subsequent induction of a variety of hepatoprotective genes in hepatocytes [27–28]. Therefore, it is curial to evaluate the effects of BM on hepatocytes including the inhibition of apoptosis and promotion of regeneration. Indeed, it is also necessary to investigate the relationship among these kinases regulated by BM in ethanol-induced hepatic injury.

In conclusion, the present study shed new light on the protective effect of BM against ethanol-induced liver injury through ameliorating hepatic inflammation. We provide the first evidence that BM reduced hepatic inflammation induced by ethanol in mice via inactivation of NF-*κ*B, STAT3 and ERK pathway. Our findings suggest that BM could potentially play therapeutic role as a novel protective agent in ALD.

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Cite this article as: LIU Xin-Yu, CHEN Guan-Nan, DU Guo-Ming, PAN Yue, SONG Wu-Qi, JIANG Ting-Wang, LIU Hai-Liang. Berbamine ameliorates ethanol-induced liver injury by inhibition of hepatic inflammation in mice [J]. *Chin J Nat Med*, 2020, **18**(3): 186-195.