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Ameliorative effect of Magnesium Isoglycyrrhizinate on hepatic encephalopathy by Epirubicin

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ABSTRACT

Background: The purpose of the present study was to evaluate the protective effect of Magnesium Isoglycyrrhizinate (MI) on Epirubicin (EPI)-induced hepatic encephalopathy (HE) and explore its underlying mechanism.

Methods: Mice were divided randomly into groups for treatments as follows: control group, EPI group (Model group), EPI + MI (25, 50 mg/kg) group. Morris water maze test were conducted to evaluate the spatial learning and memory ability. The serum and hippocampus levels of oxidative stress or inflammation were uncovered with the detection of superoxide dismutase (SOD), malondialdehyde (MDA), and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α).

Results: As a result, treatment with MI effectively ameliorated the EPI-induced decline in the ability of spatial learning and memory. MI also significantly relieved the severity of oxidative stress or inflammation in serum and hippocampus, which was accompanied with regulating liver functional parameters. Western blot data demonstrated that administration of MI could regulate the redox-related expressions of Txnip, Trx, Nrf2, HO-1, p-IκB-α, p-NF-κB, Caspase-3, Caspase-9, Bax and Bcl-2 in EPI-stimulated hepatic encephalopathy (HE). And the potency of MI treatments on Nrf2, NF-κB expression was also confirmed with immunohistochemical analysis.

Conclusions: Taken together, the protective effect of Magnesium Isoglycyrrhizinate on EPI-induced hepatic encephalopathy might be mediated via the Txnip/Nrf2/NF-κB signaling pathway.

1. Introduction

Hepatic encephalopathy (HE) is regarded as a spectrum of neurological and neuropsychiatric complication caused by severe liver disease with a high mortality index ranging from 50% to 90% [1]. It presents in two forms, chronic hepatic encephalopathy and acute hepatic encephalopathy. HE can conduce to a wide range of clinical manifestations including impaired neurological function, psychomotor dysfunction, impaired memory, sensory abnormality, poor concentration and increased reaction time [2]. The pathophysiological mechanism of HE remains poorly understood. It is generally assumed that HE is caused by an association of increased oxidative stress and inflammation.

As a member of the anthracycline family, Epirubicin (EPI) is widely used for the treatment of treat breast cancer, particularly in patients who have suffered surgical tumor excision [3]. The antitumor effect of Epirubicin on cellular proliferation has been found to rely on the combination with DNA base pairs for preventing an effective transcription during the S phase of the cell cycle [4]. However, cardiotoxicity and hepatic injury remains the main adverse effect of EPI therapy [5,6]. EPI was reported to exhibit pro-inflammatory activity, which was considered to be responsible for its hepatotoxicity [7,8]. While, it seemed a usual phenomenon that EPI treatment could disrupt a delicate equilibrium between antioxidants and oxidants in the EPI-induced liver injury, interfering mitochondria function and inducing apoptosis onset [9–11].

Therefore, we proposed a hypothesis that the hepatotoxicity of EPI might be mediated by uncontrolled reactive oxygen species (ROS) release and disturbed redox homeostasis related with mitochondria. We chose some crucial mitochondria-related proteins to assess the oxidative stress. One protein reported to be enhanced in expression by high glucose is thioredoxin-interacting protein (Txnip), also well-known as its activity of binding and inhibiting thioredoxins (Trx), one kind of pervasive antioxidant oxidase-reductase enzymes [12,13]. Txnip plays

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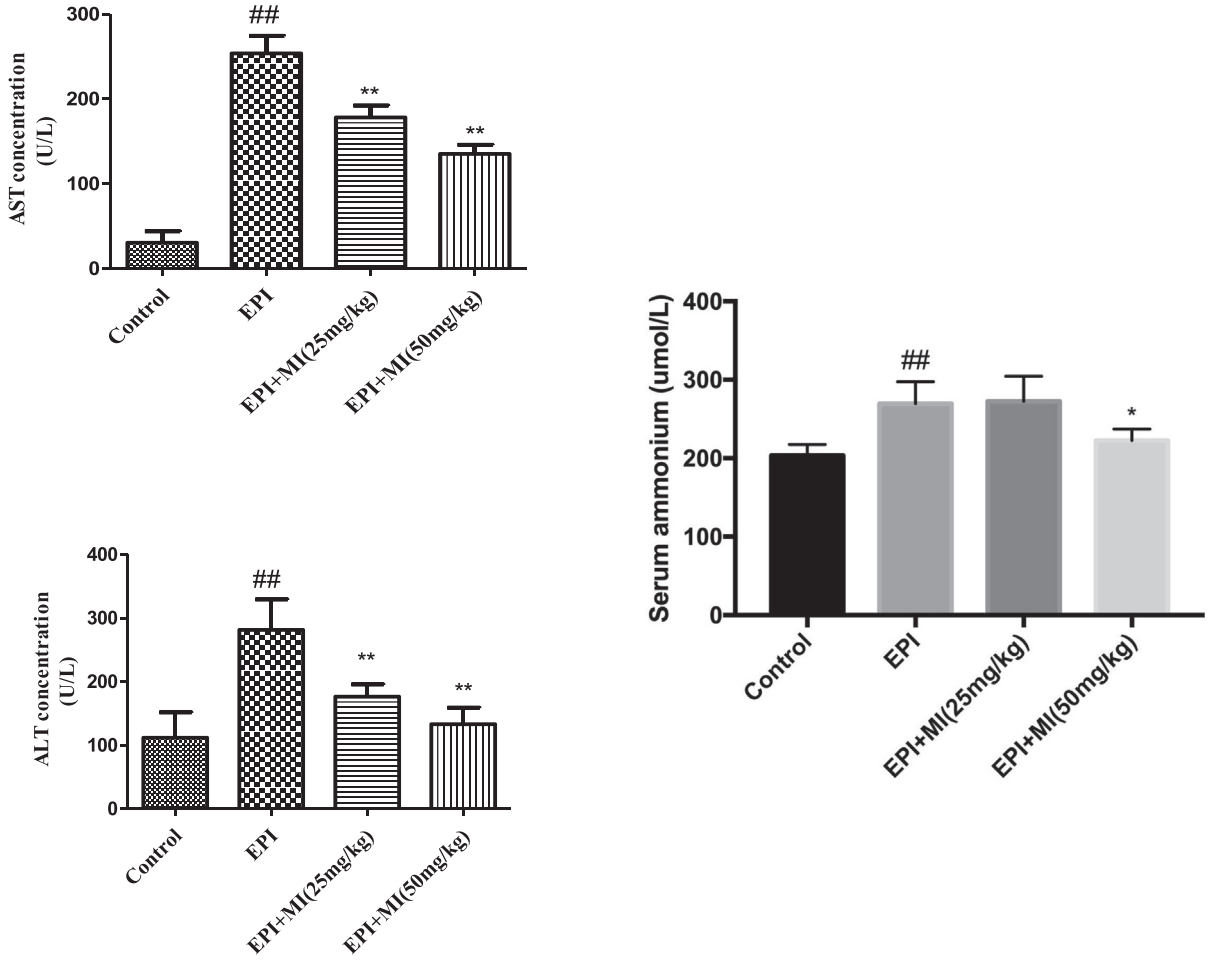


Fig. 1. Effects of MI on serum levels of ALT, AST and ammonium. The data was presented as means ± SDs. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represents means ± SDs from 6 animals. Independent experiments were performed in triplicate.

a pivotal role in diverse cellular processes, such as the regulation of proliferation, differentiation, apoptosis, cellular redox balance and inflammation [14]. Moreover, oxidative stress culminated during inflammatory responses promotes nuclear factor E2-related factor 2(Nrf2) which serves as the key effector in regulating inflammatory and oxidative responses [15]. As the critical downstream molecule, heme oxygenase (HO)-1 is an endogenous, cytoprotective enzyme which is upregulated under conditions of oxidant stress [16]. During the pathogenesis of liver failure, HO-1 and Txnip attenuates activation of nuclear factor κB (NF-κB) the generations of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α [17].

Magnesium Isoglycyrrhizinate (MI) is a magnesium salt of 18-α glycyrrhizic acid stereoisomer which can be extracted from the roots of plant *Glycyrrhiza glabra* (licorice), one of the most ancient medicinal plants. Phytochemical studies demonstrated that as the bioactive triterpene glycoside of licorice root extract, glycyrrhizic acid (GA) possessed various pharmacological potency including anti-inflammatory, antioxidative and hepatoprotective effect [18–20]. In addition, MI is currently employed as an anti-inflammatory and hepatoprotective agent in the clinical treatment of inflammatory liver diseases due to its potent effects on liver inflammatory response and liver function recovery [21,22]. However, there has been no literature to elucidate the pharmacological effect of MI on hepatic encephalopathy. Herein, the present study was to evaluate the protective effect of MI on Epirubicin-induced hepatic encephalopathy and investigate its potential mechanism.

2. Methods

2.1. Materials

Magnesium Isoglycyrrhizinate (MI) injection was purchased from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. Epirubicin (EPI) was supplied from Sigma-Aldrich (St. Louis, MO). ELISA kits of pro-inflammatory cytokines consisting of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were obtained from Shanghai Excell Biology (Shanghai, China). The alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide Dismutase (SOD) and malondialdehyde (MDA) assay kit were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Txnip (#14715), Trx (#2429), Nrf2 (#12721), HO-1 (#86806), p-IκB-α (#2859), IκB-α (#4818), p-NF-κB (#3033), NF-κB (#8242), Caspase-3 (#9664), Caspase-9 (#52873), Bax (#2772), Bcl-2 (#3498) and GAPDH (#2118) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

2.2. Animals and treatment

Male BALB/c mice (weighting 18–22 g) were obtained from Shanghai SLAC Laboratory Animal Center (Shanghai, China). Animals were allowed to acclimatize to an air-conditioned room (25 °C) under a 12 h light/dark cycle for one week before experiments with standard diet and water ad libitum. All experimental procedures were approved by the Animal Ethics Committee of China Pharmaceutical University

and carried out in accordance with the National Institutes of Guide for the Care and Use of Laboratory Animals.

Mice were divided randomly into four groups for treatments as follows: control group, EPI (9 mg/kg) group, EPI (9 mg/kg) + MI (25 mg/kg) group, EPI (9 mg/kg) + MI (50 mg/kg) group. In our study, liver injury was induced by Epirubicin (EPI) challenge in EPI group as previous report described [23]. The mice dosages of Epirubicin (EPI) were converted from clinical dosage (1 mg/kg, once/3 weeks) according to body surface area principle. And the dosage regime of Magnesium Isoglycyrrhizinate was decided by the preliminary test. MI-treated animals received intraperitoneally (i.p.) MI (25, 50 mg/kg/day) for consecutive three days followed by the intravenous (i.v.) injection of EPI (9 mg/kg) on the fourth day. Control group was given equal volume of saline along the experimental period. Thereafter, 48 h after EPI administration, one part of the mice was sacrificed by cervical dislocation after collection of serum samples. The liver, brain tissues were removed rapidly for biochemical assays or western blotting analysis. And the other part of animals received Morris water maze test training after EPI stimulation.

2.3. Morris water maze test

After EPI stimulation, Morris water maze test training started to assess spatial memory which was composed of 4-days place navigation training and a probe test on the day 5. In the present study, Morris water maze test was carried out as described in the preceding research in a black circular pool (160 cm in diameter and 50 cm in height) filled with water at approximately 25 °C [24]. The circular pool was divided into 4 quadrants, which contained a round escape platform (12 cm diameter) placed in 2.5 cm underneath the water surface. Oriented navigation trials were carried out 4 times per day for 4 consecutive days with a constant interval of 1 h and the escape latencies were recorded as Fig. 1. In each trial, the animals were gently put in water from one of the four quadrants, and the starting quadrant was also varied randomly. Within a maximum time of 90 s, animals were allowed to find the escape platform where it remained for 30 s. On day 5, another set of tests was conducted with the platform removed. Time spent in the target quadrant and the numbers of crossings over the previous target platform were recorded.

2.4. Aminotransferase and ammonium levels

The levels of ALT, AST and ammonium in serum were determined with commercial test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. The concentrations of biochemical parameters were calculated based on the standard curve.

2.5. Evaluation of oxidative stress

Hippocampus was departed from brain and lysed in ice bath for 30 min. The hippocampus supernatant was obtained after centrifugation (12,000 rpm) at 4 °C for 30 min. Then, the detection was performed to measure both the activity of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in the hippocampus supernatants and serum according to the manufacturer's instrument (Nanjing Jiancheng Biotechnology Institute, China).

2.6. Detection of pro-inflammatory cytokines by ELISA

The levels of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α in the hippocampus and serum were assayed in consistence with the protocols provided by Shanghai Excell Biology (Shanghai, China).

2.7. Immunohistochemistry analysis

The Nrf2 and NF-κB expressions in hippocampus were detected using the immunohistochemistry (IHC) analysis. The hippocampus tissues were dehydrated, embedded in paraffin, and sliced into sections. Afterwards, the paraffin sections were heated for 1 h in the oven, deparaffinized in xylene, rehydrated by graded ethanol solutions and microwaved in sodium citrate buffer. After naturally cooled to room temperature, the slides incubated in 3% hydrogen peroxide. Then the samples were incubated with primary antibody overnight at 4 °C, secondary antibody for 20 min at 37 °C and three antibody for another 20 min at 37 °C. The sections were stained by DAB coloration under the microscope and were dyed with hematoxylin (2 min). Finally, dehydration, hyalinization, and mounting were performed and the slides were observed under high magnification with picture taken in a blinded manner. Immunohistochemistry analysis was quantified using image-j software in order to assess effects.

2.8. Western blot

Protein extraction and western blot analysis were conducted as the research described [25]. Hippocampal protein was obtained with the RIPA lysis buffer (Beyotime, China) followed by the assay of protein extracts contents using the BCA kit (Beyotime, China). Briefly, samples containing approximately 50 mg protein were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by the transference to polyvinylidene fluoride membranes (Millipore Corporation, MA, USA). And then blots were blocked with 5% skim milk in Tris buffer saline-Tween20 (TBST) for 2 h. And then the membranes were incubated overnight with primary antibodies diluted at 1:1000 at 4 °C followed by incubation with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling Technology Inc., USA). GAPDH (1:1000) was used as the invariant control. The membranes were visualized by the enhanced chemiluminescence detection system (Pierce, USA) and quantification of relative intensities were examined using Quantity One (Bio Rad). Representative blots photographs were obtained from three independent experiments.

2.9. Statistical analysis

All data were expressed as mean values ± standard deviations. Differences between groups were analyzed by two-way ANOVA with the Tukey multiple comparison test and Bonferroni correction. The P-values < 0.05 could be acceptable as a significant change. Statistical analysis was carried out using GraphPad Prism 5.0 vision.

3. Results

3.1. MI treatment improved liver function

Activities of ALT and AST in serum were measured to elucidate the degree of hepatocyte damage. As revealed in Fig. 1, serum AST, ALT levels of EPI group maintain the highest concentration compared with those of control group, suggesting hepatotoxicity in mice was caused by administration of EPI exposure. However, mice treated with MI exhibited a marked reduction of ALT and AST activities, with more significant reversion after treatment with MI (50 mg/kg). Meantime, the hepatoprotective potency of MI was verified by the relieved ammonium concentration in comparison with ones of EPI group.

3.2. MI treatment suppressed cognitive impairment induced by EPI

The spatial influence was assessed via Morris water maze test. As shown in Fig. 2, during the 4-day place navigation training, animals in the EPI group had apparent impairment in spatial learning ability, as evidenced by the longer escape latency compared to the control ones. Intriguingly, treatment with MI exhibited the shorter escape latency than those of EPI-stimulated group.

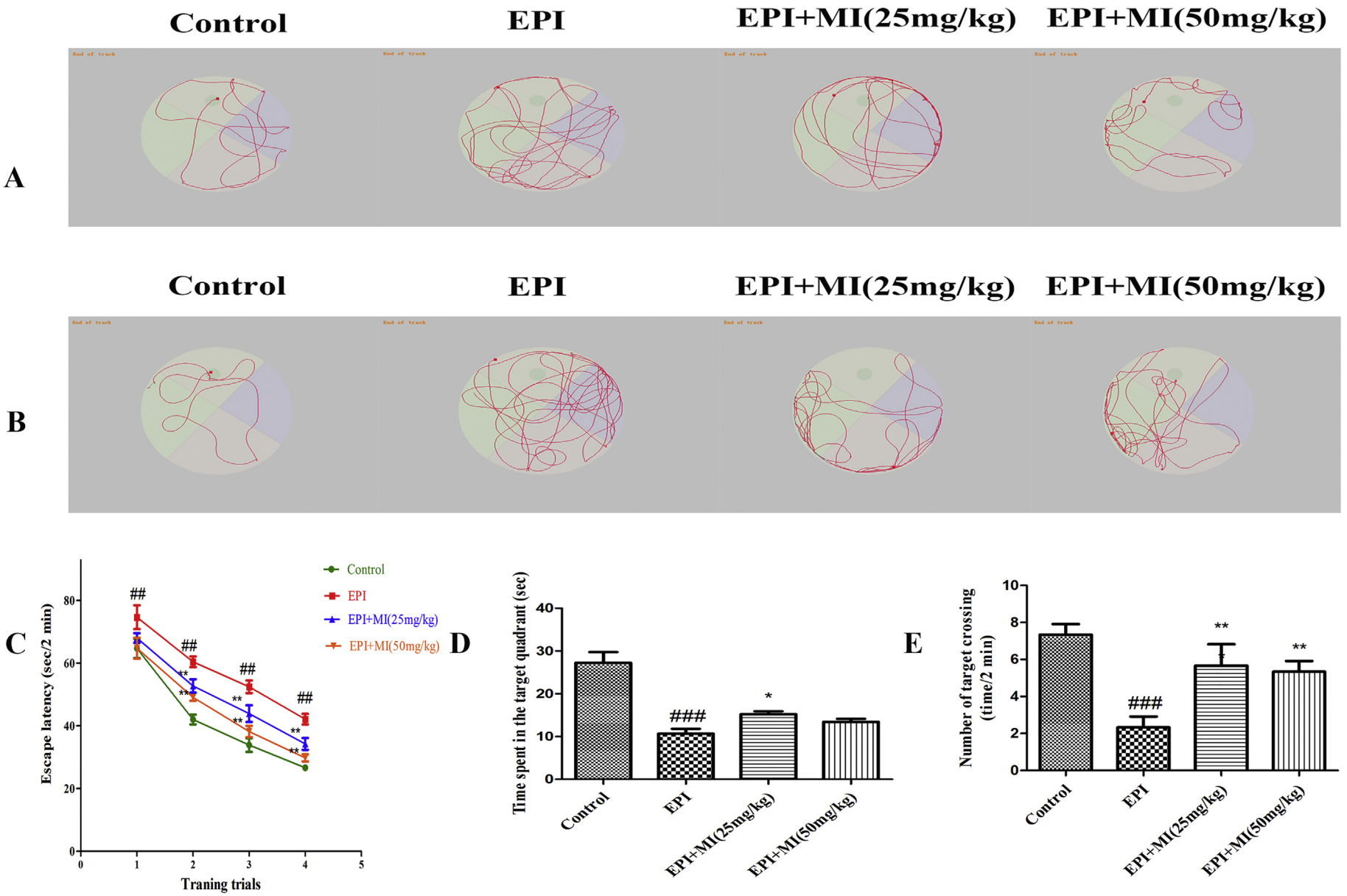


Fig. 2. Effects of MI on the EPI-induced cognitive impairment in Morris water maze test. (A) Representative searching strategy of mice on the second day after EPI challenge. (B) Representative searching strategy of mice on the fifth day after EPI challenge. (C) Escape latency to find the hidden platform in the test within 2 min during the five consecutive days. (D) The time spent in the quadrant where the platform was once placed within 90s. (E) Number of target crossing within 2 min. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n = 5 for each group.

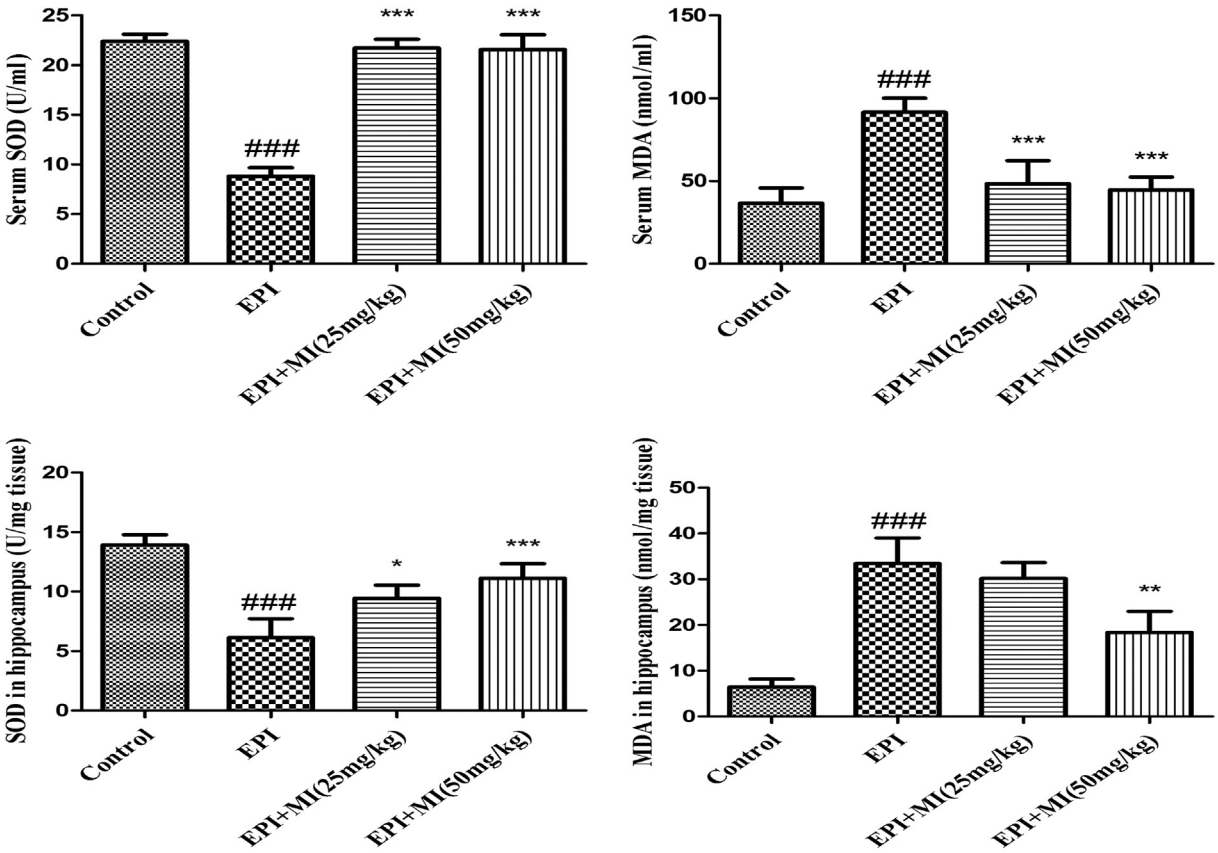


Fig. 3. Effects of MI on the levels of SOD and MDA in serum and hippocampus. The data was presented as means ± SDs. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represents means ± SDs from 6 animals. Independent experiments were performed in triplicate.

Afterwards, mice were subjected to the probe trial where the target platform was removed to further investigate the effect on brain cognition ability. Mice showed the decreased numbers of crossings over the previous target platform and increased time spent on target quadrant in contrast with control mice. While, MI-treated EPI mice displayed striking increases in the target crossing numbers compared to merely EPI-treated mice, suggesting that MI could restore the cognitive impairment caused by EPI administration.

3.3. MI treatment inhibited oxidative stress in serum and hippocampus

To determine lipid peroxidation triggered by EPI, we measured the levels of SOD, MDA in serum and hippocampus. As illustrated in Fig. 3, we observed the marked decrease in SOD activity as well as the obvious increase in MDA content after EPI challenge in contrast with control group, whereas these changes above in serum and hippocampus were restored significantly by the MI treatment (25, 50 mg/kg).

3.4. MI treatment down-regulated TNF-α, IL-6 and IL-1β levels in serum and hippocampus

Neuroinflammation in serum and hippocampus were determined by detecting alterations in the serum and hippocampal levels of cytokines. As expected, the current study revealed remarkable increase of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β in serum or hippocampus, while MI (25, 50 mg/kg) treatment exhibited an apparent reversion of TNF-α, IL-6 and IL-1β to control levels, compared with those in EPI group. Meantime, it was noteworthy that somehow MI (25 mg/kg) administration seemed to possess a more favorable potency than GM (50 mg/kg) treatment in the serum contents of pro-inflammatory cytokines, which was opposite with those in the hippocampal cytokine levels (Fig. 4).

3.5. Effect of MI on the expressions of Nrf2 and NF-κB in hippocampus by immunohistochemical analysis

The expressions of Nrf2 and NF-κB were also evaluated by immunolabeling. As depicted in Figs. 5 and 6, as compared with the control group, EPI challenged caused the lower expression of Nrf2 and higher expression of NF-κB. On the contrary, the treatment with MI notably increased the protein level of Nrf2 and decreased the protein level of NF-κB in EPI-induced hepatic encephalopathy.

3.6. Effect of MI on expression levels of Nrf-2/HO-1/Trx/Txnip/NF-κB signaling pathway

To explore whether MI could lead to upstream alterations, we utilized western blotting analysis to measure the protein expression of Nrf-2, HO-1, Trx/Txnip and NF-B signaling pathway. Exposure to EPI pronouncedly inhibited the protein expressions of Nrf2, HO-1 and Trx. In addition, protein expressions of Txnip, p-NF-κBp65 and p-IκBα in hippocampus of EPI-treated animals were dramatically up-regulated in contrast with the control ones. However, MI treatment significantly reversed EPI effects on alterations of Nrf-2/HO-1/Trx/Txnip axis, IκBα depletion and NF-κB activation in the hippocampus of MI animals (Fig. 7).

3.7. Effect of MI on expression levels of apoptosis-related proteins

To assess the effect of MI on EPI-induced hepatotoxicity, apoptosis-related protein including Bcl-2 protein family members (Bax and Bcl-2), caspase-3 and caspase-9 were assayed by Western blot analysis.

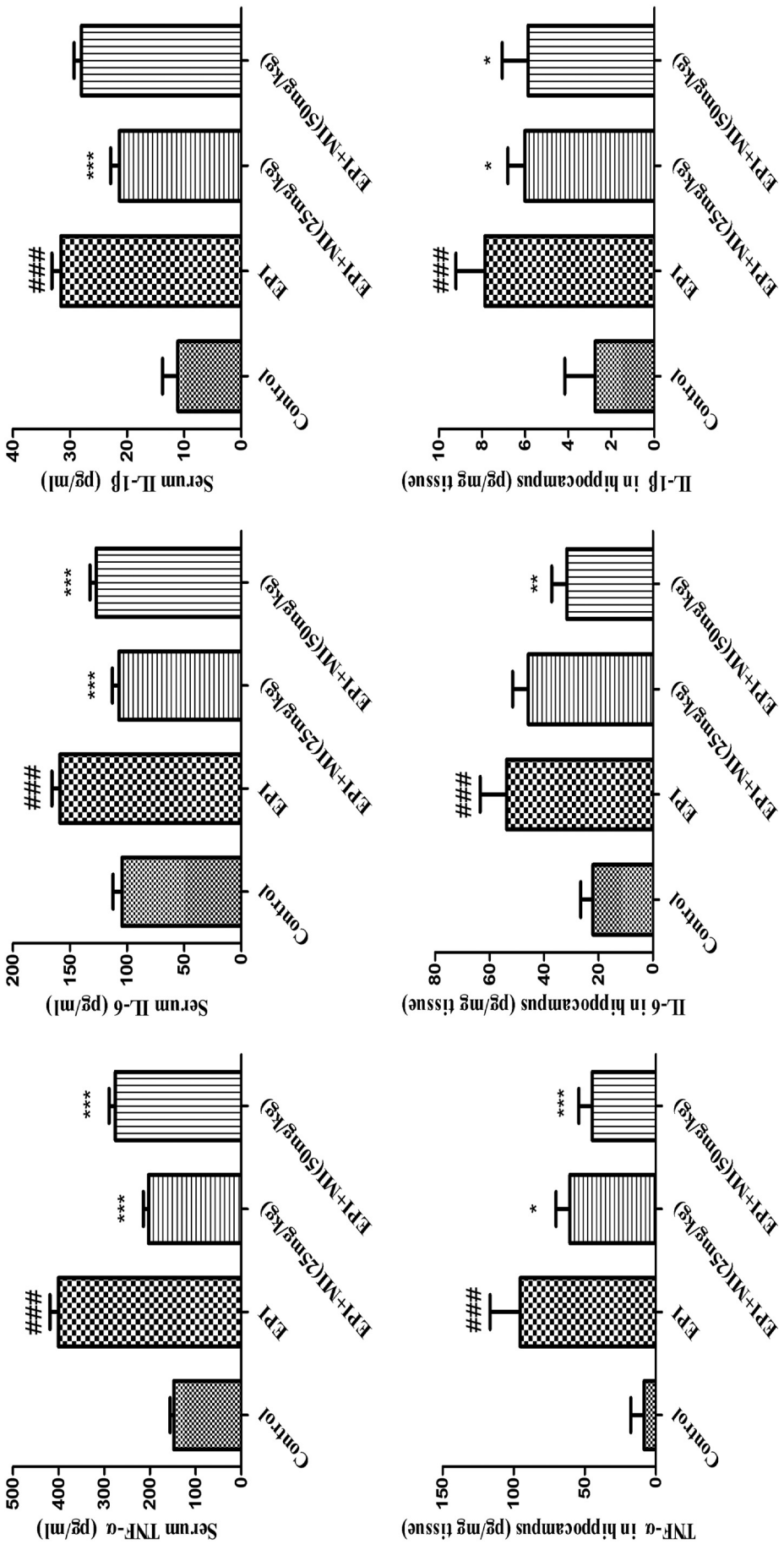


Fig. 4. Effects of MI on the contents of IL-6, IL-1β and TNF-α in serum and hippocampus. The data was presented as means ± SDs. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represents means ± SDs from 6 animals. Independent experiments were performed in triplicate.

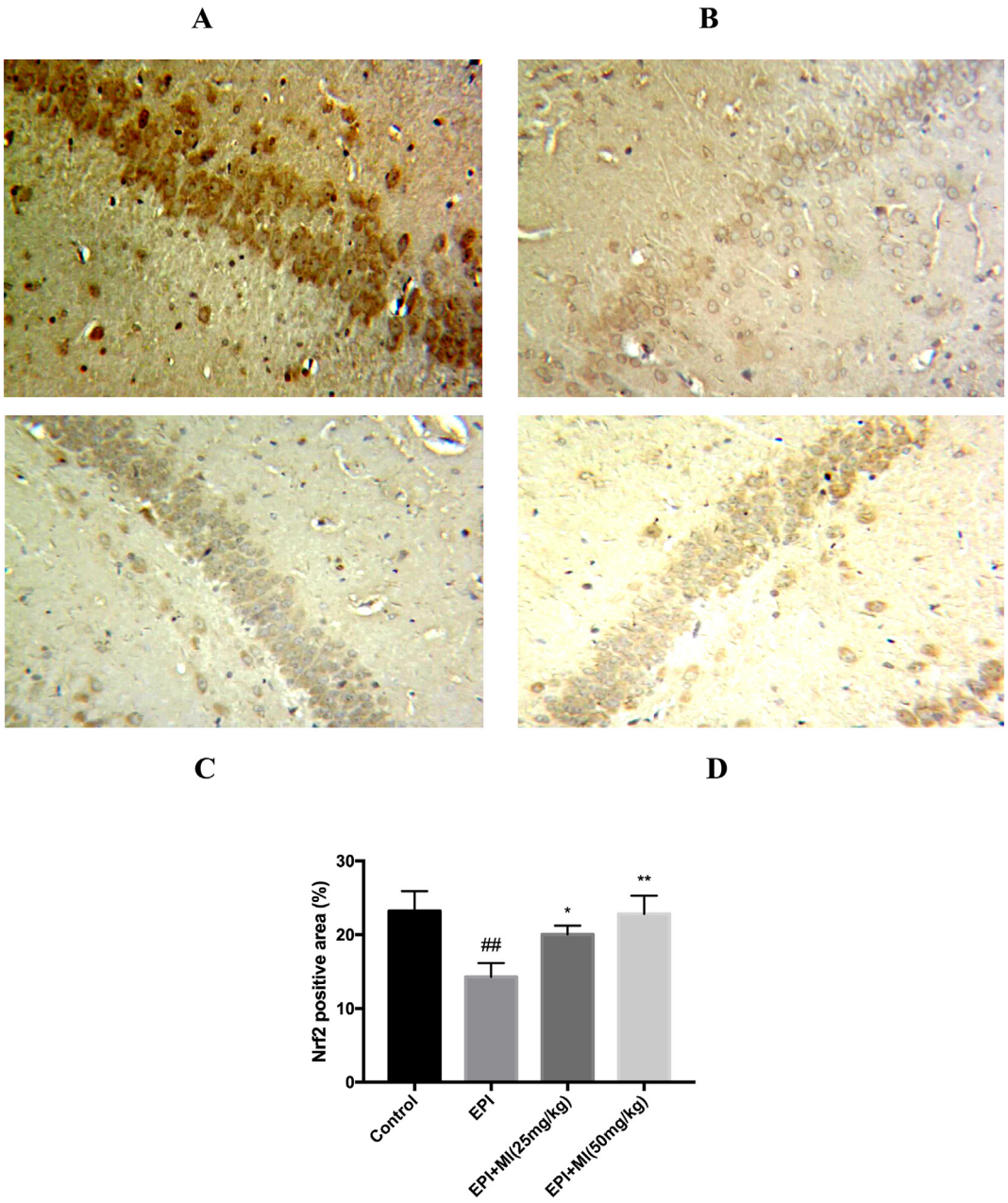


Fig. 5. Effects of MI on the expressions of Nrf2 in hippocampus by immunohistochemical analysis. Data shown are representative photographs from each treatment group of mice. A: the control group; B: the EPI group; C: the EPI + MI (25 mg/kg) group; D: the EPI + MI (50 mg/kg) group. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n = 3 for each group. Independent experiments were performed in triplicate.

Quantification of Western blot showed that EPI stimulation caused the notable augment in Bax/Bcl-2 ratio as well as caspase-3/caspase-9 activation in EPI-treated hippocampus in comparison with control hippocampus. As expected, MI administration effectively inhibited the apoptosis process by declining the Bax/Bcl-2 ratio and caspase-3/caspase-9 level as depicted in Fig. 7.

4. Discussion

EPI is a widely used antineoplastic which increases the cure rate decreases the recurrence rate of breast cancer [26]. However, its clinical application is limited by considerable acute and chronic normal heart and liver toxicity. Primarily, EPI is metabolized to generate the epirubicinol and EPI glucoronide in liver tissues, which are associated with oxygen free radicals-related hepatic toxicity [9]. Several piece of evidence indicated that MI exhibited the hepatoprotective effect in hepatic lipotoxicity and hepatic ischemia/reperfusion injury [27,28].

AST and ALT activities are the key biochemical markers to reveal the condition of hepatic injury. The results showed that treatment with MI effectively reduced the activities of transaminase, which confirmed that MI was beneficial for the liver dysfunction caused by EPI. Neurobehavioral abnormalities are the major clinical consequences of chronic HE. Poor performance was observed in the MWM test in arthritis-induced cognitive decline. MI stimulation considerably reduced the escape latency of HE mice in the MWM test, suggested that MI might enhance hippocampal-mediated learning and memory abilities.

MDA is a reliable indicator sensitive to oxidative damage [29]. SOD defends against ROS by catalyzing and decomposing O_2 radicals and H_2O_2 [30]. Numerous evidence proved the involvement of SOD and MDA in the cognition deficits and hepatic disorder [31]. In our study,

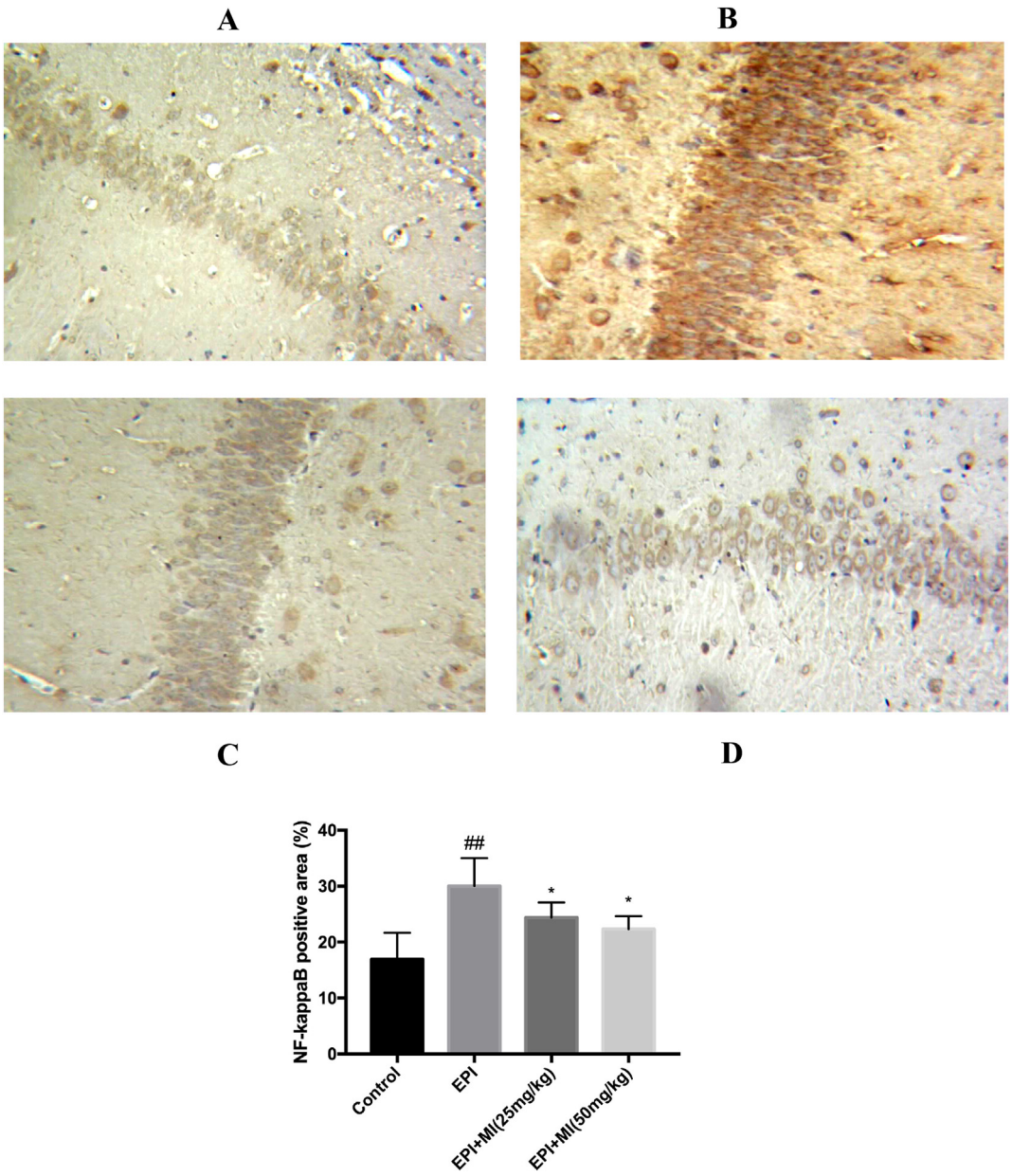


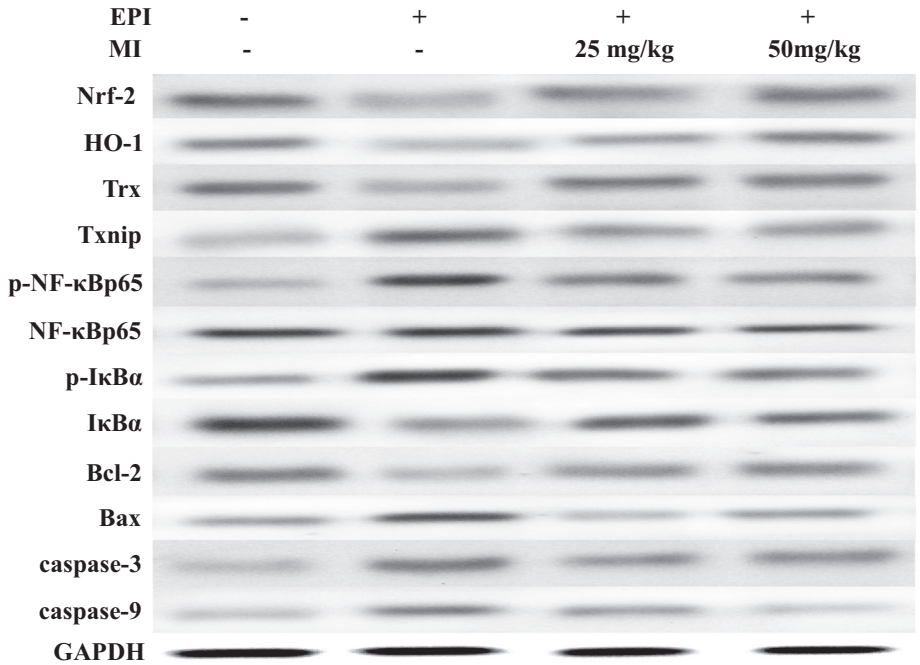
Fig. 6. Effects of MI on the expressions of NF-κB in hippocampus by immunohistochemical analysis. Data shown are representative photographs from each treatment group of mice. A: the control group; B: the EPI group; C: the EPI + MI (25 mg/kg) group; D: the EPI + MI (50 mg/kg) group. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n = 3 for each group. Independent experiments were performed in triplicate.

the significant increase in MDA level and the reduction of SOD activity induced by EPI suggested that lipid peroxidation caused liver tissue injury. By contrast, treatment with MI decreased the formation of MDA and restored the activity of SOD.

It was proposed that pro-inflammatory cytokines were important modulators by initiating, amplifying and perpetuating the inflammatory response in the liver injury [32]. The inflammatory cytokines are also involved in the depressive-like behavior [33]. Additionally, increased IL-1β, IL-6 and TNF-α levels were observed in brains of animals with acute hepatic encephalopathy [34]. Our data indicated that the apparent effect of glycyrrhizic acid on HE might take place in the liver because studies on tissue distribution of glycyrrhizic acid reported an extremely low concentration of glycyrrhizic acid in the brain. However, the liver status could have an impact on the cerebral function by regulating endogenous metabolites, such as blood ammonium. Therefore, the protective effect of glycyrrhizic acid on neurological parameters might be mainly derived from its hepatoprotective property.

The hippocampus is acknowledged as a site of plasticity during the acquisition of spatial memory. Thioredoxin-interacting protein (Txnip), namely vitamin D3-upregulated protein-1 or Trx-binding protein-2, is generally acknowledged as an inhibitor of Trx. Txnip inhibits Trx ability by interacting with a number of other cellular molecules [35]. Txnip plays an important role in the meditation of oxidative stress during the pathogenesis of brain damage and hepatic injury [36,37]. The western blot analysis revealed that MI treatment markedly suppressed the Txnip activity and enhanced the Trx activity in response to EPI challenge.

As to our knowledge, reactive oxygen species (ROS), causes oxidative damage to nearby cells, which in turn activates Nrf2 and initiates Nrf2-dependent gene expression [38]. As the downstream element, antioxidant response elements (ARE) signaling pathway is considered to play a pivotal role in the upregulation of cellular antioxidant enzymes



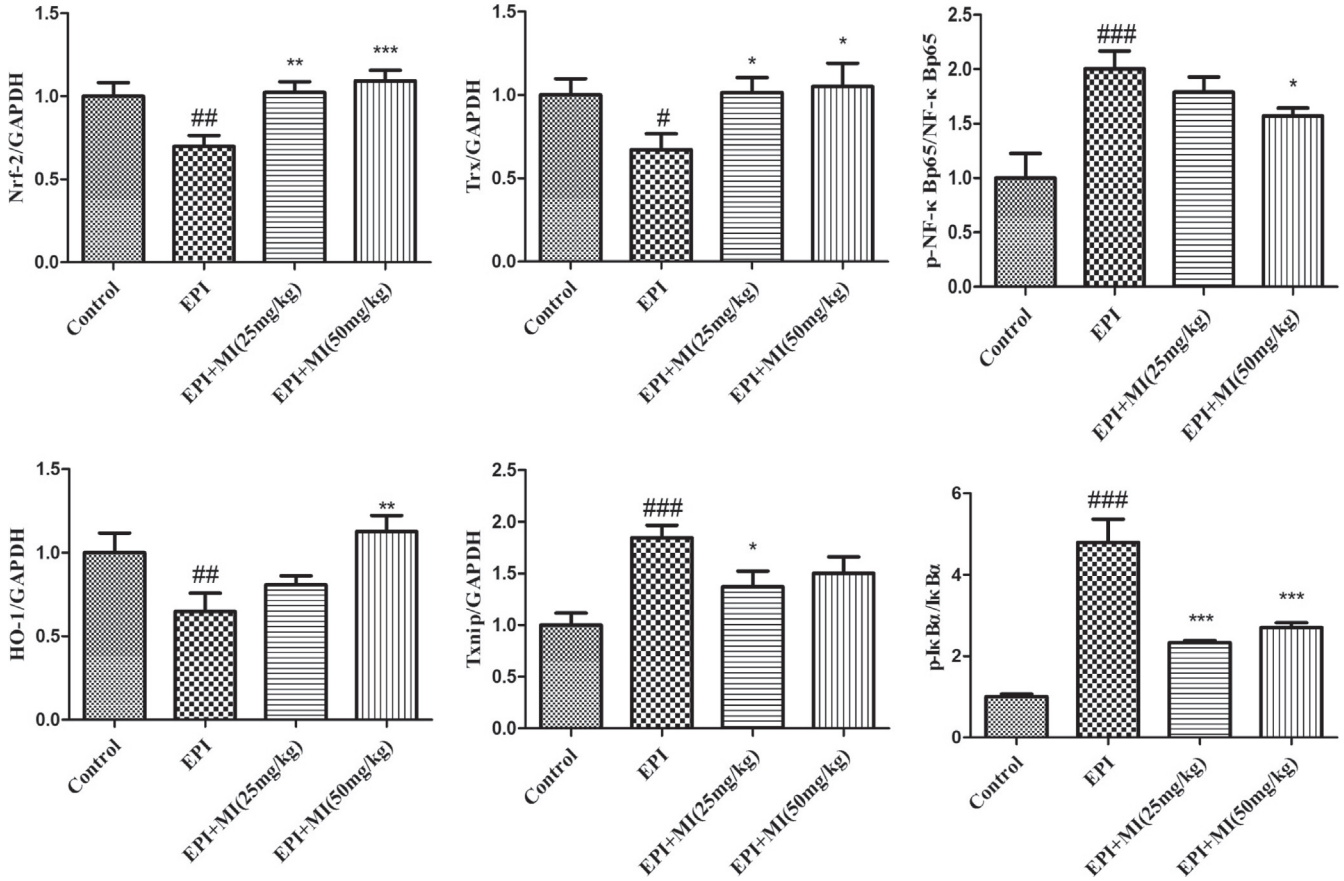


Fig. 7. Effects of MI on the expression levels of Nrf-2/HO-1/Trx/Txnip/NF-κB axis as well as apoptosis-related proteins in hippocampal region. Compared with control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Compared with EPI group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represents each group of random 3 samples. Independent experiments were performed in triplicate.

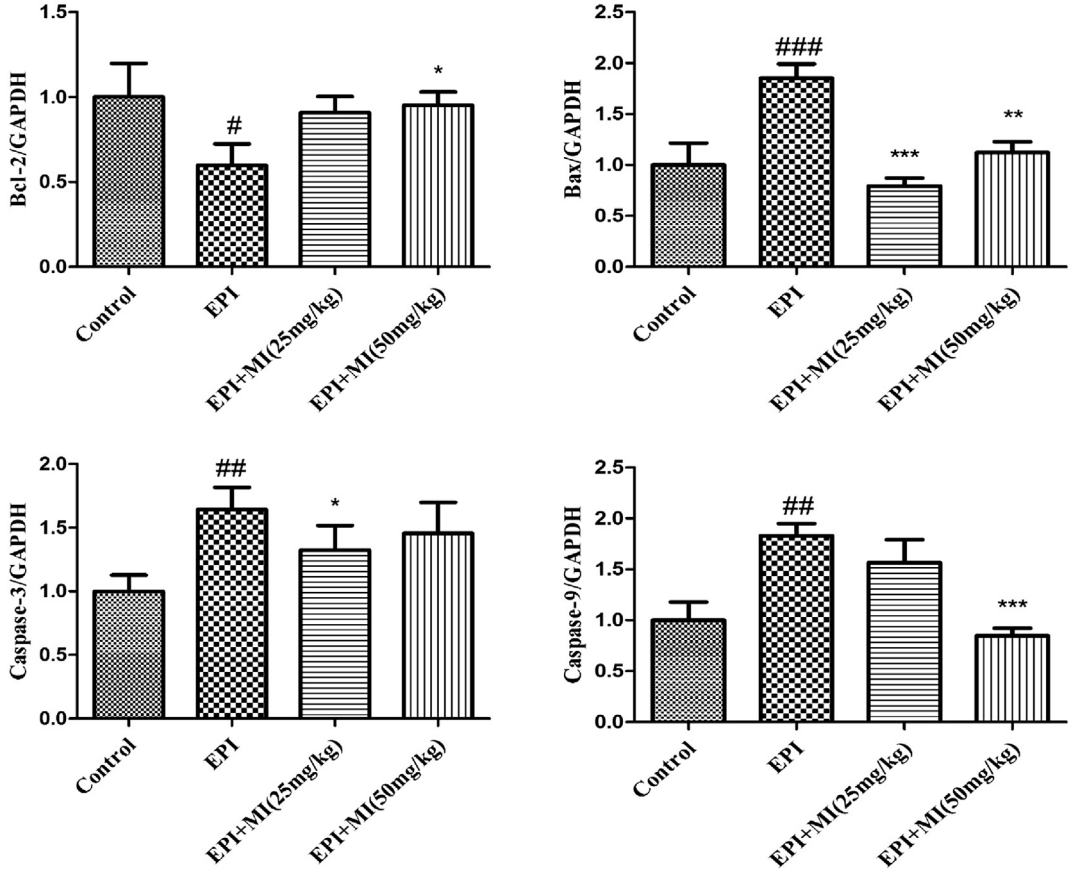


Fig. 7. (*continued*)

including heme oxygenase-1 (HO-1). HO-1 is a potent endogenous cytoprotective enzyme promoted by a variety of inflammatory stimuli and cellular oxidative stresses [39]. HO-1 drives the phosphorylation and degradation of IκB-α, which further contributes to the activation of NF-κB [40]. NF-κB is the essential molecule governing the transcription of downstream target including inflammatory cytokines and oxidative events [41]. Our results showed that MI could restore the Nrf2/HO-1 expression and inhibit the phosphorylation of NF-κB caused by EPI stimulation.

Caspases are cysteine proteases which specifically promoted during apoptotic process. The harmful stimulus activates caspase-9, which then activates effector caspase-3 and leads to apoptosis. Former literatures indicated that caspase-3/9 served as critical mediators of apoptosis in liver injury [42]. Meanwhile, The Bcl-2 family of proteins such as anti-apoptotic proteins (Bcl-2) and proapoptosis proteins (Bax) regulate programmed apoptosis [43]. Several investigators pointed out that the over-expression of anti-apoptotic protein Bcl-2 could reduce hepatic encephalopathy [44]. MI administration significantly down-regulated the relative expression of caspase-3, caspase-9, Bax and up-regulated the expression of Bcl-2. Our data implied that MI could inhibit the apoptotic reaction in EPI-challenged hepatic encephalopathy.

In conclusion, the present study demonstrated that treatment with MI effectively attenuated the transaminase activity and cognitive decline in EPI-induced hepatic encephalopathy. The potential mechanism might be related to the anti-inflammatory, anti-oxidative and anti-apoptotic properties through the Txnip/Nrf2/NF-κB pathway. Further researches are warranted before clinical application.

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