

# Neuroprotective effect of berberine against learning and memory deficits in diffuse axonal injury

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**Abstract.** The aim of the present study was to assess the neuroprotective effect of berberine against learning and memory deficits in diffuse axonal injury (DAI). DAI rats were orally gavaged with berberine at a dose of 200 mg/kg of body weight for 4 weeks. Behavioral tests were used to analyze the neuroprotective effect of berberine against DAI-induced learning and memory deficits. In the present study, treatment with berberine significantly protected against DAI-induced inhibition of learning and memory in rats. Notably, berberine significantly suppressed the levels of tumor necrosis factor, interleukin-1 $\beta$  and monocyte chemoattractant protein-1, as well as reduced the protein expression levels of nuclear factor- $\kappa$ B, Bcl-2-associated X protein and cytochrome *c* in DAI rats. In addition, berberine significantly suppressed the protein expression of p38 mitogen-activated protein kinase, activating transcription factor 2 and vascular endothelial growth factor in DAI rats. These results suggested that berberine exhibited a neuroprotective effect against learning and memory deficits in severe DAI through the suppression of inflammation, angiogenesis and apoptosis in a rat model.

## Introduction

In recent years, the worldwide morbidity of traumatic brain injury has increased, and in North America alone, traumatic brain injury is estimated to affect 1.7 million individuals per year (1). As a common disease of the central nervous system, the high mortality and disability rates resulting from diffuse

axonal injury (DAI) are a great public health threat and financial burden for the families of patients, as well as the society (2). Clinically, intracranial hemorrhage and edema can be quickly acknowledged through computed tomography and magnetic resonance imaging (3). However, as the severity of injury and individual tolerance of patients differ, demonstrations of vital signs are quite distinctive in each case. Therefore, intracranial metabolic abnormalities and the degree of injury should be evaluated on a case-by-case basis, in order to offer individualized therapy and reduce rates of mortality and disability (4).

Generally, DAI is accompanied by injury of the endangium and destruction of the blood-brain barrier (BBB), resulting in hypoxia-ischemia of injured brain tissues. Thus, this injury results in DAI, which aggregates dysneuria (5). Subsequent to DAI, brain tissue regions reconstruct the capillary network to recover the blood supply of the ischemic cerebrum (6).

In recent years, various clinical observations and animal experiments have suggested that berberine is an effective treatment for diabetes mellitus type 2 (7). It can significantly regulate blood sugar levels and improve impaired glucose tolerance. In addition, it has preferable preventive and therapeutic effects against diabetic complications, such as cardiovascular disease, hypertension and hyperlipidemia (8). As an isoquinoline derivative alkaloid, berberine exists in *Coptischinensis*, golden cypress and the root of Chinese barberry (8). The chemical structure of berberine is shown in Fig. 1. A previous study observed that berberine has the pharmacological functions of regulating blood sugar levels, reducing blood fat content, reducing blood pressure and inhibiting aldose reductase (9). These studies have indicated that berberine may have a favorable clinical value and prospect as a preventive therapy for diabetes (10,11). In the present study, the neuroprotective effect of berberine against learning and memory deficits in severe traumatic brain injury was investigated.

## Materials and methods

**Animals and treatment groups.** All procedures were approved by the Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China). Male Sprague-Dawley rats (10-11 weeks-old, 260-300 g, n=30) were housed in pairs with a 12/12 h light/dark cycle at 22 $\pm$ 2°C and 55 $\pm$ 5% humidity,

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and standard rat chow and water were provided *ad libitum*. All Sprague-Dawley rats were randomly assigned to one of following three groups (n=10): Sham, DAI model and berberine group. Rats of the berberine group were orally gavaged with 200 mg/kg of body weight berberine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 weeks. Rats of the sham and DAI model groups were orally gavaged with normal saline. Rats of the sham group were anesthetized with an intraperitoneal injection of 3% sodium pentobarbital (60 mg/kg).

**DAI model.** A DAI rat model was constructed as previously described (12). Briefly, rats were anesthetized with 3% sodium pentobarbital (60 mg/kg) by intraperitoneal injection. The dorsal surface of the skull was exposed using a midline incision, and a steel disc was fixed centrally between the lambda and bregma regions using dental cement. The laterally extended plate containing the foam bed was restricted to a horizontal position. Next, the rat was placed at a prone position and two belts were fixed at the trunk of the rat, while a third belt was fixed at the head of the rat to maintain the alignment between the center of rotation of the head and the rotational bearing. A Plexiglas tube was positioned directly above the cranium. Subsequently, three successive impacts were delivered at 10-min intervals by dropping a 450 g weight on the cranium. At 24 h after establishment of the DAI models, the course of berberine treatment was initiated for rats in the berberine group.

**Learning and memory test.** The learning and memory of rats was investigated by a Morris water maze test. A circular tank (23±1°C) was used, which was filled with water that was made opaque and included a variety of extra-maze cues. Rats were habituated to the water and apparatus prior to water maze testing for 5 min/day for 5 days. In the spatial acquisition phase of the test, a platform (50x50x50 cm) was submerged in the water with an extra-maze cue (90x90x40-cm square container, 60-cm distance from the platform) for 10 min and their movements were automatically tracked (SMART 3.0; Panlab, Barcelona, Spain). A transparent lucite platform was submerged at 2 cm below the water surface, which was remained at this position for all spatial trials. All rats were subjected to 4 trials/day (1 trial/start position with the start positions being each corner of a 3x2 cm<sup>2</sup> parameter surrounding the platform) for 4 consecutive days. The rats were given 60 sec to locate the submerged platform and remained there for 30 sec. The escape latencies (in sec) and path lengths (in cm) were recorded. For each rat, consecutive trials were initiated immediately after removal of the rat from the platform. Following the spatial acquisition phase of the trial, a daily 60-sec probe trial was used to evaluate how well the rats had learned the location of the platform. The swimming time was recorded inside a 1.8-m diameter pool in which the platform was centrally located.

**Measurement of tumor necrosis factor (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and monocyte chemoattractant protein-1 (MCP-1) levels.** After treatment with berberine, rats were anesthetized with 30 mg/kg pentobarbital intraperitoneal injection (i.p.; Sigma-Aldrich; Merck KGaA) and venous blood was collected from the eye socket of each rat and centrifuged at 4,000 x g for 5 min at 4°C. According to the manufacturer's instruction

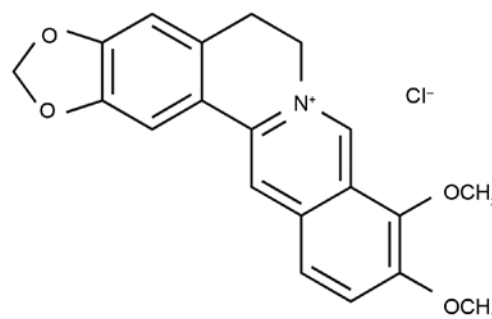


Figure 1. Chemical structure of berberine.

of ELISA kits (ExCell Bio, Shanghai, China), the serum was collected and used to analyze the concentrations of TNF- $\alpha$  (ER006-48), IL-1 $\beta$  (ER008-96) and MCP-1 (EM018-96).

**Western blot assays.** The protein levels of nuclear factor (NF)- $\kappa$ B, Bcl-2-associated X protein (Bax), cytochrome c, p38 mitogen-activated protein kinase (p38 MAPK), activating transcription factor 2 (ATF-2) and vascular endothelial growth factor (VEGF) were determined by western blot analysis. Briefly, rats were sacrificed by decollation after the final round of Morris water maze tests under 30 mg/kg pentobarbital (i.p.) and hippocampal tissue was immediately isolated. Protein was extracted from the samples using radioimmunoprecipitation assay buffer containing protease inhibitor (RIPA, Beyotime Institute of Biotechnology, Haimen, China) at 4°C for 30 min, followed by centrifugation at 12,000 x g for 10 min at 4°C. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, 50 ng total protein was fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked at 37°C with 5% non-fat dry milk for 30 min and incubated with the appropriate primary antibody, including anti-NF- $\kappa$ B (1:1,000; 8242), anti-Bax (1:1,000; 5023), anti-cytochrome c (1:1,000; 11940), anti-p-p38 MAPK (1:2,000; 4511), anti-ATF-2 (1:2,000; 5112), anti-VEGF (1:2,000; 9698) and anti- $\beta$ -actin (1:2,000; 4970; all from Cell Signaling Technology, Inc., Beverly, MA, USA) at 4°C for 12 h. Membranes were subsequently incubated with secondary antibody conjugated with horseradish peroxidase (sc-2357; 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at 37°C and detected with enhanced chemiluminescence reagents (EMD Millipore). The protein expression was scanned and quantified by densitometric analysis using an image analyzer Quantity One System 3.0 (Bio-Rad Laboratories, Inc., Richmond, CA, USA).

**Statistical analysis.** Data are presented as the means  $\pm$  standard deviation. Statistical analysis used the Student's t-test to assess statistical differences. For all test, a difference with  $P < 0.05$  was considered as statistically significant.

## Results

**Neuroprotective effect of berberine against learning and memory deficits in DAI.** To examine the effect of berberine

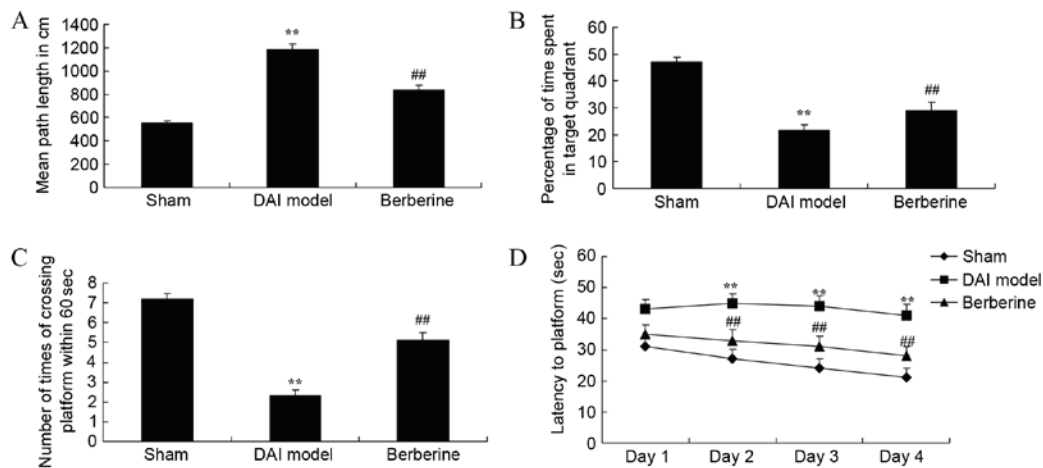


Figure 2. Neuroprotective effect of berberine treatment (200 mg/kg) against learning and memory deficits in DAI. (A) Mean path length, (B) time spent in the target quadrant, (C) times crossing the platform, and (D) escape latency were investigated. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury.

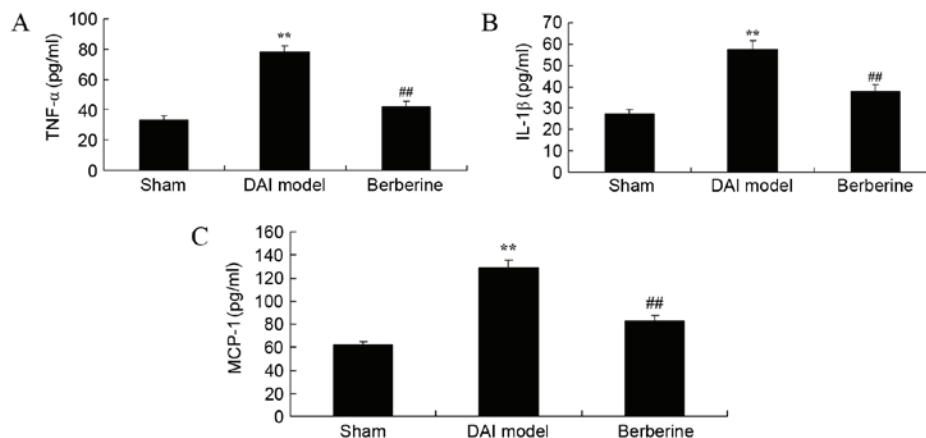


Figure 3. Neuroprotective effect of berberine on (A) TNF- $\alpha$ , (B) IL-1 $\beta$  and (C) MCP-1 levels in DAI rats. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; MCP-1, monocyte chemoattractant protein-1.

on the learning and memory in DAI rats, behavioral tests were used. The results demonstrated that the mean path length of the DAI model group was higher compared with that of the sham group (Fig. 2A). Next, the percentage of time spent in the target quadrant and the number of times of crossing the platform within 60 sec were lower in DAI model group in comparison with those of the sham group (Fig. 2B and C). In addition, the time of latency to platform was also higher in the DAI model group than in the sham group (Fig. 2D). However, treatment with berberine significantly improved the results of the behavioral tests in DAI rats, when compared with the untreated DAI model group (Fig. 2A-D).

**Effect of berberine on TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 levels in DAI.** To study the anti-inflammatory effects of berberine in DAI rats, the concentrations of TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 were surveyed using ELISA kits. The results revealed that the concentrations of TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 were significantly increased in the DAI model group in comparison with those of the sham group (Fig. 3). However, treatment with berberine significantly reduced the levels of TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 in DAI rats, indicating an anti-inflammatory effect (Fig. 3).

**Neuroprotective effect of berberine against NF- $\kappa$ B/p65 protein expression in DAI.** To investigate the mechanism of berberine against the inflammation induced by DAI, the NF- $\kappa$ B/p65 protein expression was detected using western blot assay. The results demonstrated that DAI significantly induced the NF- $\kappa$ B/p65 protein expression in rats, compared with the sham group (Fig. 4). However, berberine pretreatment significantly suppressed the protein expression of NF- $\kappa$ B/p65 in the DAI rats (Fig. 4).

**Neuroprotective effect of berberine against Bax protein expression in DAI.** To further investigate the molecular mechanism of the action exerted by berberine against DAI, the Bax protein expression was analyzed in the current study. As shown in Fig. 5, a significant increase in Bax protein expression induced by DAI was observed, when compared with the sham group (Fig. 5). Pretreatment with berberine, however, significantly suppressed Bax protein expression in DAI rats (Fig. 5).

**Neuroprotective effect of berberine against cytochrome c protein expression in DAI.** To explore the molecular mechanism of berberine against DAI, cytochrome c protein expression was also analyzed. Fig. 6 demonstrates that there was a significant

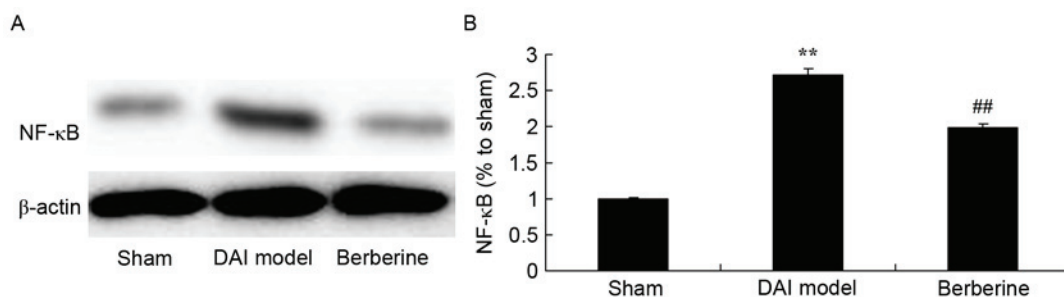


Figure 4. Neuroprotective effect of berberine against NF- $\kappa$ B protein expression in DAI rats, as observed in the (A) western blots and (B) quantified results of NF- $\kappa$ B protein expression. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

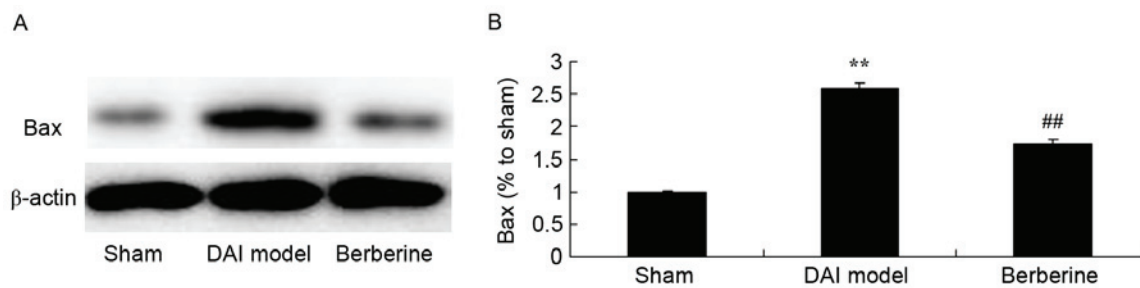


Figure 5. Neuroprotective effect of berberine against Bax protein expression in DAI rats, as observed in the (A) blots and (B) quantified results of western blot analysis. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; Bax, Bcl-2-associated X protein.

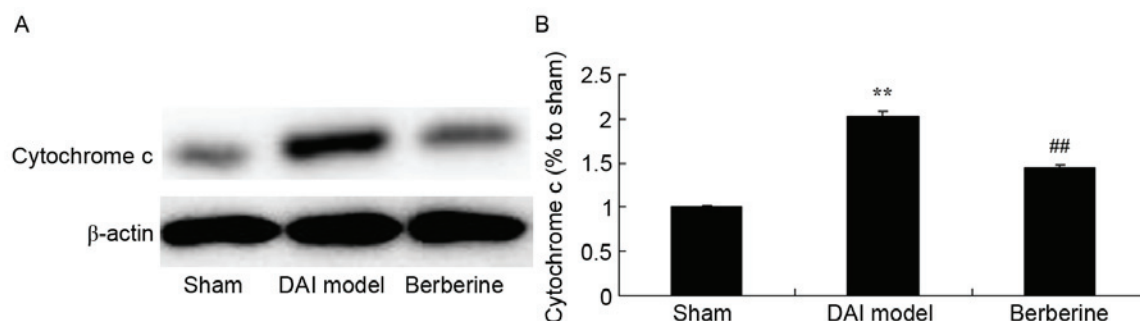


Figure 6. Neuroprotective effect of berberine against cytochrome *c* protein expression in DAI rats, as observed in the (A) blots and (B) quantified results of western blot analysis. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury.

increase in cytochrome *c* protein expression of DAI group rats, compared with the sham group. Administration of berberine significantly weakened the protein expression of cytochrome *c* in DAI rats (Fig. 6).

Neuroprotective effect of berberine against p38 MAPK protein expression in further explore the molecular mechanism of berberine against DAI, p38 MAPK was investigated as an important pathway in the effect of berberine. The results of western blot assays showed that p-p38 MAPK protein expression in the DAI model group was higher than that of the sham group (Fig. 7). Treatment with berberine significantly suppressed the protein expression of p-p38 MAPK in DAI rats (Fig. 7).

**Neuroprotective effect of berberine against reduced ATF-2 protein expression in DAI.** To further study the effect of berberine on ATF-2 protein expression in DAI, western blot analysis was performed. As shown in Fig. 8, the expression

of ATF-2 protein was significantly suppressed by DAI, as compared with the sham group. However, berberine treatment significantly recovered the protein expression of ATF-2 in the DAI rats (Fig. 8).

**Neuroprotective effect of berberine against reduced VEGF protein expression in DAI.** The effect of berberine on VEGF protein expression in DAI rats was further examined using western blot assay. Compared with the sham group, the induction of DAI was found to markedly suppress the expression of VEGF protein in the model group (Fig. 9). Treatment with berberine significantly recovered the protein expression of VEGF in DAI rats (Fig. 9).

## Discussion

The functions of extensive cerebral contusion and laceration, and high intracranial pressure subsequent to DAI result in



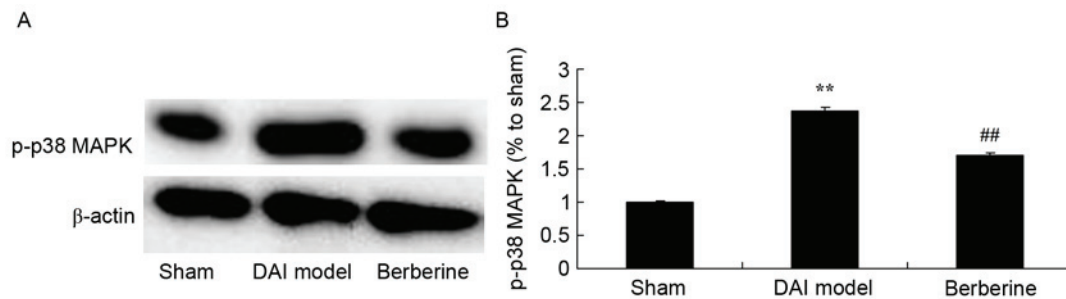


Figure 7. Neuroprotective effect of berberine against p38 MAPK protein expression in DAI rats, as observed in the (A) blots and (B) quantified results of western blot analysis. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; MAPK, mitogen-activated protein kinase.

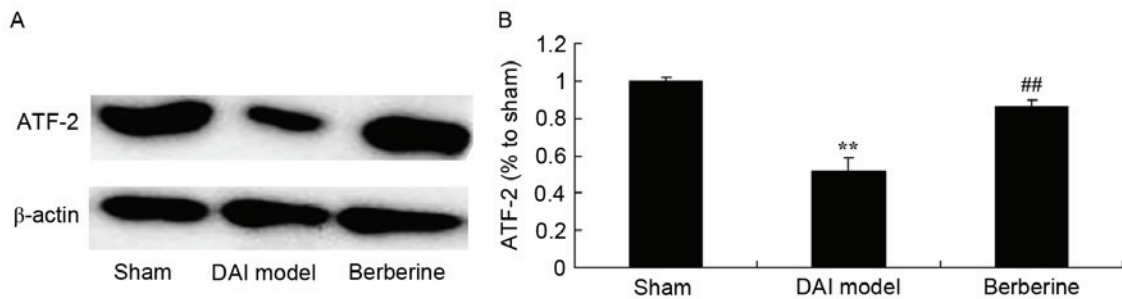


Figure 8. Neuroprotective effect of berberine against inhibition of ATF-2 protein expression in DAI rats, as observed in the (A) blots and (B) quantified results of western blot analysis. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; ATF-2, activating transcription factor 2.

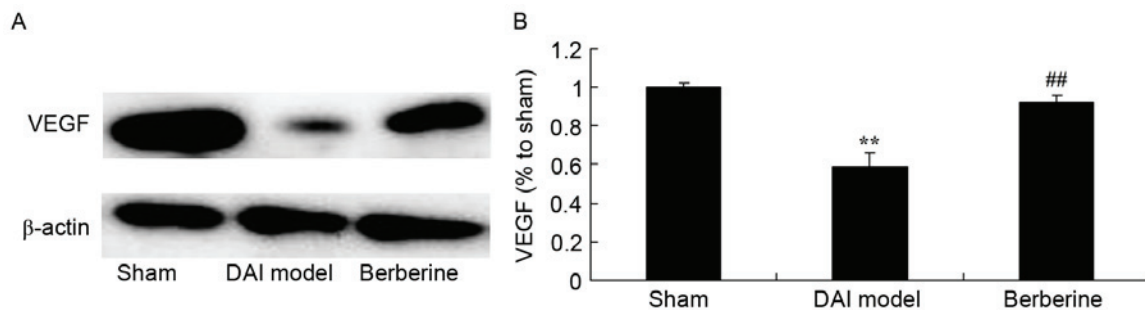


Figure 9. Neuroprotective effect of berberine against inhibition of VEGF protein expression in DAI rats, as observed in the (A) blots and (B) quantified results of western blot analysis. \*\* $P < 0.01$  vs. sham group; ## $P < 0.01$  vs. DAI model group. DAI, diffuse axonal injury; VEGF, vascular endothelial growth factor.

increased production of pro-inflammatory cytokines, while the inflammatory cascade reaction is initiated, which triggers secondary cerebral lesion, cerebral metabolic changes and brain death (13). It has been proven that, after cerebral lesion, the concentrations of plasma  $\text{TNF-}\alpha$ , IL-6 in DAI and IL-1 $\beta$  are increased (14). Meanwhile, encephal edema, hyperglycemia and subarachnoid hemorrhage further aggravate DAI and destroy the BBB. This promotes the expression of biological effects of pro-inflammatory mediators and inflammatory mediators, including the increase of neutrophil granulocytes, fever and the increase of endothelial permeability (15). The present study demonstrated that treatment with berberine significantly improved learning and memory in DAI rats. Certain studies have suggested that berberine protects against brain ischemia through regulatory effects on the Akt/glycogen synthase kinase 3 $\beta$  and extracellular signal-regulated kinase 1/2 survival and apoptotic signaling pathways, respectively (16,17). Thus, the inhibitory effect of

berberine may improve DAI-induced learning and memory deficits in rats.

Monocytes, macrophages, endothelial cells and contractile fiber cells express MCP-1 (18,19). The major biological effects of MCP-1 are the chemotaxis of monocytes and that it can act on lymphocytes and basophilic granulocytes, while it has no biological effect on neutrophil granulocytes. MCP-1 receptor is a member of the g-protein coupled receptor super-family (18). Following the combination of MCP-1 and its targeted specific receptor, the receptor of MCP-1 is activated, thus activating the phosphoinositide 3-kinase pathway through g-protein coupling on the cytomembrane (20). Meanwhile, MCP-1 activation may trigger the release of calcium ion in cytoplasm and induce the activation of protein kinase C. Subsequent to DAI, injured cerebral tissues produce various inflammatory chemokine factors. One of these factors is MCP-1, which is a major inflammatory chemokine factor that induces strong chemotaxis to monocytes/macrophages (18). Monocytes/macrophages

gather in inflammatory response regions and participate in the occurrence and progression of inflammatory response in DAI. Xu *et al* (9) demonstrated that berberine attenuated cigarette smoke-induced TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 expression in mice. The present study showed that berberine significantly reduced the concentrations of TNF- $\alpha$  and IL-1 $\beta$ , and also reduced MCP-1 concentration, in DAI rats. These results suggested that berberine had an anti-inflammation effect in DAI rats.

NF- $\kappa$ B is a group of transcription factors of eukaryotes, which is widely distributed in the nervous system. When cells are stimulated, the phosphorylation and ubiquitylation are initiated through a second messenger system. NF- $\kappa$ B and I $\kappa$ B are activated and shifted into the karyon from the cytoplasm. It has been observed that NF- $\kappa$ B was activated in rats with DAI at the early stages of injury (21). Activated NF- $\kappa$ B was identified in the cytoplasm and cell nucleus of neurons after 24 h of injury. During the acute inflammatory reaction process, NF- $\kappa$ B participates in the activation of macrophages and hemamoeba, and controls the genetic expression of proinflammatory factors (22). Controlling this process would lead to the amplification of inflammatory responses and tissue injuries (23). Chen *et al* reported that berberine protects against neuronal damage via suppression of inflammation and TLR4/MyD88/NF- $\kappa$ B signaling in traumatic brain injury (11). In the present study, berberine pretreatment was found to significantly suppress the protein expression of NF- $\kappa$ B/p65 in DAI rats.

The activities of p38 MAPK are significantly increased in microglial cells. Activated p38 MAPK is located in the karyon or endochylema, which is possibly associated with the functions and status of microglial cells. In addition, p38 MAPK expression is correlated with the activities of NF- $\kappa$ B (24), thus, a p38 MAPK inhibitor may block the transcription of NF- $\kappa$ B. Major biological functions after the activation of p38 MAPK include generation and activation of various inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 (25). Macrophages at an ischemic core region may present activated p38 MAPK, indicating that p38 MAPK may participate in the inflammatory responses when cerebral ischemic injury occurs (26). Activated p38 MAPK can enter into the cell nucleus or shift to other regions (27), and may further activate various transcription factors, including ATF-2/6, ATH-1/2, ETS21, MAX, HSF21, myocyte enhancer binding factor-22, nuclear transcription factor 2P, CHOP/GADD153, Elk-1 and SAP-1 (28). A study by Liu *et al* indicated that berberine reduces fibronectin and collagen accumulation through the p38 MAPK signaling pathway in rat glomerular mesangial cells (29). The present study also observed that berberine significantly suppressed the protein expression levels of cytochrome *c* and p-p38 MAPK in DAI rats.

VEGF is selectively distributed among the endochylema and cell membrane of vascular endothelial cells, and is highly expressed in the traumatic and tumor growth processes (30). VEGF is known to be the strongest antagonist, which can increase the permeability of endothelial cells. It promotes angiogenesis and is a key regulatory factor (24), while also promoting the proliferation of endothelial cells. In addition, VEGF induces endothelial cells to express proteolytic enzyme, interstitial collagenase and tissue factors. The current study observed that berberine activated ATF-2 and VEGF protein expression in DAI rats. Similarly, Tsang *et al* demonstrated

that berberine suppressed the growth and development of lung metastases through HIF-1 $\alpha$ /VEGF signaling in hepatocellular carcinoma (10).

In conclusion, the present study confirmed that berberine exerted a neuroprotective effect in DAI rats by improving learning and memory, and suppressing inflammation. This neuroprotective action resulted from inhibition of NF- $\kappa$ B/p65, cytochrome *c* and p-p38 MAPK levels, as well as activation of the ATF-2 and VEGF signaling pathway. Thus, these findings suggest that berberine may be a potential drug in the prevention of DAI in clinical practice.

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