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Protective effects of 8-MOP on blood-brain barrier via the Nrf-2/HO-1 pathway in mice model of cerebral infarction

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Abstract. – OBJECTIVE: To explore the effect of 8-MOP on the blood-brain barrier in mice model of cerebral infarction and the underlying mechanism.

MATERIALS AND METHODS: Middle cerebral artery occlusion (MCAO) model was established to induce permanent cerebral infarction. The neurological function was observed and scored by the modified longa score method after model establishment. Besides, the water content of brain tissue was measured by the standard dry weight method. Evans blue exudation rate was used to evaluate the effect of 8-MOP on the permeability of the blood-brain barrier. Western-blot and quantitative polymerase chain reaction (qPCR) were used to detect the expression of MMP-9, claudin-5, vascular endothelial growth factor (VEGF), as well as the NFE2-related factor 2 (Nrf-2)/hemeoxygenase 1 (HO-1) pathway.

RESULTS: 8-MOP could reduce the neurological deficit scores in a dose-dependent manner, thereby reducing cerebral edema. After 8-MOP treatment, the expression of MMP-9 decreased in ischemic brain tissue, whereas the expression of claudin-5, VEGF, and GFAP increased, suggesting that the blood-brain barrier ultrastructure was improved. In addition, the expression of Nrf-2 and HO-1 decreased after the model establishment of cerebral infarction. However, the expression of Nrf-2 and HO-1 increased in ischemic brain tissue after 8-MOP treatment.

CONCLUSIONS: 8-MOP may protect the blood-brain barrier via the Nrf-2/HO-1 pathway.

Key Words:
Cerebral infarction, Blood-brain barrier, 8-MOP, Nrf-2/HO-1.

Introduction

Ischemic stroke is the most common type of stroke. Assessment of burden of disease, injury, and risk factors worldwide has shown that cerebral infarction is the second most common cause of death, and the third most common cause of disability[1,2]. The incidence, mortality, disability rate, and recurrence rate of cerebral infarction have been increasing year by year[3]. Cerebral infarction is characterized by long recovery time and poor prognosis, which brings a heavy burden for both society and families. During cerebral infarction onset, there is a marked decrease in oxygen content and rapid necrosis of the nervous tissue in the core area of the infarct, while the peripheral infarct area exhibits a modest reduction in oxygen content and perfusion, which can be rescued[4-6]. There is a risk of infarction in this peripheral area but no irreversible damage has yet been established. Therefore, the ischemic penumbra is the focus of stroke revascularization and neuroprotective therapy currently. Intravenous thrombolysis is considered to be the most effective treatment for acute cerebral infarction. However, the thrombolysis rate after cerebral infarction is not so high due to strict treatment time and post-treatment hemorrhage complications[7]. Therefore, it is particularly important to find new targets for drug therapy and to provide a reliable basis for the treatment of cerebral infarction.

The blood brain barrier (BBB) is a special barrier that exists between the cerebral blood circulation and nervous tissue. Its main function is to maintain the brain tissue homeostasis, regulate the balance of brain material exchange, and protect the brain tissue from damage[8,9]. The pathophysiological process of ischemic stroke involves the BBB dysfunction, as well as the primary and secondary destruction of cells and cell death. Since the blood brain barrier plays a vital role in the pathogenesis of the cerebrovascular disease, it is now considered as a new drug target for the treatment of cerebral infarction. Meanwhile, studies

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on the blood-brain barrier may provide a basis for clinical drug research.

The nuclear transcription factor Nrf-2 (NFE2-related factor 2, Nrf-2) binds to cell pressure signals and regulates the expression of several downstream detoxifying enzymes as well as antioxidant enzymes by participating in the transcription process[10]. The Nrf-2/HO-1 (heme oxygenase 1, HO-1) signaling pathway is one of the most important cellular defense mechanism[11]. As a protective factor for the brain, Nrf-2 plays a vital part in the inflammatory response and oxidative stress after cerebral infarction[12]. After cerebral infarction, there are a large number of reactive oxygen species (ROS) in cells, which contribute to oxidative stress and the release of cytotoxic substances[13]. However, drug-activated Nrf-2/HO-1 signaling pathway can initiate intracellular anti-oxidative stress response after cerebral infarction, suggesting the Nrf-2 signaling pathway has cytoprotective effects[14]. Studies have shown that in the brain injury model, Nrf-2 agonist sulforaphane could activate Nrf-2 signaling pathway and increase the integrity and stability of the blood brain barrier. Therefore, Nrf-2 signaling pathway and its downstream protective factors are key targets for the treatment of cerebral blood-brain barrier[15].

8-MOP (8-methoxypsoralen) is a photochemotherapeutic agent commonly used in conjunction with ultraviolet (UV)[16]. UV-combination therapy often referred to psoralen ultraviolet therapy (PUVA), is effective for the treatment of skin diseases such as psoriasis, vitiligo, and T-cell lymphoma[17,18]. In psoralen ultraviolet therapy, 8-MOP can be activated into a DNA reactive compound by UV photosensitization, thereby obtaining anti-proliferative and cytotoxic activity[19]. Previous studies have been mostly focused on the role of 8-MOP in combination with UV. So far, no systematic study has been conducted on the effect of 8-MOP on cerebral vascular barrier during cerebral infarction, especially its direct effect on endothelial cells.

In this work, middle cerebral artery occlusion (MCAO) model was established to induce permanent cerebral infarction by the modified longa thread embolectomy[20]. We aimed to investigate whether 8-MOP could protect the blood brain barrier in the mice model and explored its regulatory mechanisms. We hoped to provide new evidence and experimental basis for the development and clinical application of 8-MOP.

Materials and Methods

Animals

Adult male CD-1 mice between 10-12 weeks weighing 27-30 g were purchased from the Animal Management Center of Yangzhou University (Yangzhou, China). Mice were housed under 12:12 h light-dark cycle with the temperature controlled between 20-25°C and the humidity controlled at 60 ± 5%. This study was approved by the Animal Ethics Committee of The Third People's Hospital of Qingdao Animal Center.

Establishment of Mice MACO Model

Middle cerebral artery occlusion (MCAO) mice model was established according to the modified Longa methods[20]. CD-1 mice were randomly divided into 2 groups: sham operation group and the operation group. The operation group was further divided into 4 groups: the MOP group was intraperitoneally injected with different doses of 8-MOP (5 mg/kg, 10 mg/kg, and 15 mg/kg) while the vehicle group was intraperitoneally injected with an equal volume of saline 2 h after occlusion. The neurological behavior of mice was observed and scored 24 h after model establishment (n=10 in each group). Besides, the water content of brain tissue was measured by the standard dry weight method (n=6).

Neurological Function Evaluation

According to the Longa neurological deficit scoring method[20], neurological deficit evaluations of mice were performed after mice woke up from anesthesia for 24 h. Neurological deficit symptoms were recorded and graded on a scale of 0-5 (0 point: no symptoms of nerve damage; 1 point: unable to stretch the contralateral forelimbs; 2 points: contralateral forelimb flexion; 3 points: mild contralateral circling; 4 points: severe contralateral cycling; 5 points: fall to the opposite side), in which higher score represented more severe injury. The investigators observing neurological functions were blinded to the group allocation.

Evans Blue Staining Assay

22 hours after the MCAO model establishment, mice were injected with 2% Evans Blue (4 mL/kg) through the tail vein. After 2 hours, mice were anesthetized and the heart was perfused with about 50 mL saline to wash away Evans Blue in other parts of the brain and then were decapitated. The frontal cortex and cerebellum were removed from the brain using a mouse grinder. After that, the brain was cut into 5 slices and photographed

in order. Then, the brain was divided into the infarct hemisphere and non-infarct hemisphere, which were collected in different EP tube and homogenized with 1 mL trichloroacetic acid. After centrifuged for 20 minutes, the supernatant was collected for the measurement of absorbance with a microplate reader.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) and complementary Deoxyribose Nucleic Acid (cDNA) was synthesized by reverse transcription. Relative gene expressions were detected by Real-time quantitative PCR.

The sequences of the primers are listed below: MMP-9: forward 5'-CGTCTCGGGAAGGCTCTGCTGTT-3'; reverse 5'-GGCAGAAATAGGCTTTGTCTTGGTA-3'; Claudin-5: forward 5'-AAAGGCACGGGTAGCACTC-3'; reverse 5'-TCATAGAACTCGCGGACAACG-3'; VEGF: forward 5'-ATCATGCGGATCAAACCTCACC-3'; reverse 5'-GGCTTTGTTCTGTCTTTCTTTGGTC-3'; GFAP: forward 5'-GGAGTGGTATCGGTCTAAGTTTGC-3'; reverse 5'-GTTGGCGGCGATAGTCGTTAG-3'; β-actin: forward 5'-GCCTTCCTTCTTGGGTAT-3'; reverse 5'-GGCATAGAGGTCTTTACGG-3'

Western Blotting

Total proteins were extracted by radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). Proteins were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) while the immunoblots were incubated overnight at 4°C with the diluted primary antibodies. After washed in Tris-buffered saline and tween (TBS-T) for 3 times, the immunoblots were incubated with fluorescence-labeled secondary antibody for 1h at room temperature. After washed in TBS-T for 3 times, protein bands were scanned and detected by Odyssey far-infrared fluorescence scanning imaging system.

Statistical Analysis

Statistical analysis was performed by the Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare multiple sets of data. The S-N-K test was performed to compare differences between groups. The data were represented as mean ± SEM. $p<0.05$ was considered statistically significant.

Results

Effect of 8-MOP on Neurological Deficit in Mice Model of Cerebral Infarction

The neurological deficits in mice were determined by modified Longa scoring method. Results showed that the neurological impairment score in the vehicle group was significantly higher than that in the sham group, indicating that the permanent middle cerebral artery infarct model caused a significant neurological deficit. In addition, the neurological deficit scores in the 8-MOP treatment group were significantly lower than the vehicle group (Figure 1A), suggesting that

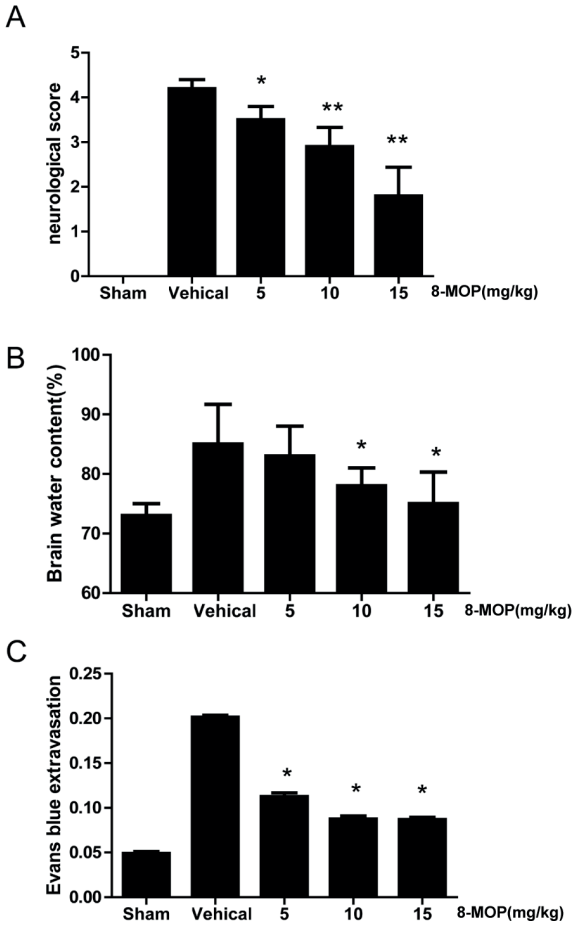

Figure 1. Effect of 8-MOP on cerebral ischemic brain tissue. ***A,*** Mice treated with different doses of 8-MOP and mice in the vehicle group were scored on their neurological function (*$p<0.05$ compared with the vehicle group). ***B,*** The brain water content was measured in mice treated with different doses of 8-MOP or an equal volume of saline (*$p<0.05$ compared with the vehicle group). ***C,*** The blood brain barrier permeability was detected by the Evans blue exudation assay in mice treated with different doses of 8-MOP or an equal volume of saline (*$p<0.05$, **$p<0.01$ compared with vehicle group).

8-MOP treatment could improve the neurological function in a cerebral infraction.

Effect of 8-MOP on Cerebral Water Content in Mice Model of Cerebral Infarction

The water content of brain tissue was measured by a dry weight method. The results showed that the water content of the damaged brain tissue in the operation group was significantly increased compared to the sham operation group. Besides, the water content of brain tissue in the 10-mg/kg and 15-mg/kg 8-MOP treatment group was significantly lower 24 h after modeling than the vehicle group ($p<0.05$). However, no significant difference was observed between the water content of mice treated with 5 mg/kg 8-MOP or an equal volume of saline ($p>0.05$) (Figure 1B), demonstrating that 8-MOP could decrease the cerebral water content in a dose-dependent manner.

8-MOP Reduces Blood-Brain Barrier Permeability in Experimental Cerebral Infarction Mice

Next, we investigated the effect of 8-MOP on blood-brain barrier permeability by the Evans blue exudation rate assay. As shown in Figure 1C, there was no Evans Blue exudation in the sham group and the blood-brain barrier was intact in these mice. However, mice in the operation group which were injected with saline (vehicle group) showed severe Evans Blue exudation after 24 hours. 8-MOP treatment markedly decreased the Evans Blue exudation rate comparing to the vehicle group at 24 hours ($p<0.05$), which indicated that 8-MOP could reduce blood-brain barrier permeability and maintain its integrity. Based on these above findings about the neurological deficit score, cerebral edema and Evans blue exudation rate, we found that a high dose (15.0 mg/kg) and medium dose (10.0 mg/kg) of 8-MOP could achieve ideal treatment effect. Therefore, the follow-up study was performed at a middle dose of 8-MOP (10.0 mg/kg).

8-MOP Can Inhibit the Degradation of Tight Junction Proteins

Tight junction protein dysfunction is an important aspect of the pathogenesis of cerebral infarction. In MCAO rats, downregulated expression of tight junction proteins including claudin-5, occluding and zonula occludens1 (ZO-1) can increase the permeability of the blood brain barrier[14]. In addition, it has been found that MMP-9 is correlated with the cerebral infarct area[16]. Therefore, we examined the protein and mRNA expression of MMP-9 and claudin-5. Results showed that the expression of MMP-9 in the 8-MOP treatment group was significantly decreased ($p<0.05$) (Figure 2A, B and D) than the vehicle group. Meanwhile, the expression level of claudin-5 was significantly increased at 24 h ($p<0.05$) (Figure 2A, 2C and 2E). These results demonstrated that 8-MOP could inhibit the degradation of tight junction proteins and reduce the redistribution of tight junction proteins.

8-MOP Stabilizes Endothelial Cell Function and the Astrocyte Membrane Skeleton

Endothelial cells, astrocytes, pericytes, and extracellular matrix constitute the skeleton of the blood brain barrier, all of which provide support for the special structure of the blood brain barrier. Vascular endothelial growth factor (VEGF) is a key factor in promoting angiogenesis. As shown in Figure 3 A and C, 8-MOP treatment significantly increased the protein and mRNA levels of VEGF at 24 h comparing to those in the vehicle group ($p<0.05$) (Figure 3A, 3B and 3C). Glial fibrillary acidic protein (GFAP) is a marker of astrocyte activation. It is also one of the components of the astrocyte skeleton and maintains the tightness of the astrocyte membrane. Western-blot and qRT-PCR results showed that 8-MOP treatment significantly increased the protein expression and mRNA levels of GFAP at 24 h ($p<0.05$ compared with the vehicle group) (Figure 3D, 3E and 3F). These above finding revealed that 8-MOP could initiate angiogenesis after infarction and protect the function of endothelial cells. Besides, 8-MOP could stabilize the blood brain barrier structure, thereby maintaining its function and increasing its stability.

8-MOP Increases Nrf-2 and HO-1 Expression

Nrf-2, as a brain protective factor, plays a crucial role in the regulation of inflammation and oxidative stress after cerebral infarction[6]. In this study, we explored whether 8-MOP protected the blood brain barrier *via* Nrf-2. Results showed that the expression of Nrf-2 in the 8-MOP group was significantly increased at 24 h compared with the vehicle group ($p<0.05$) (Figure 4A and 4B). Since Nrf-2 is the most important transcription factor of HO-1, we also detected the expression of HO-1 by Western blotting and qRT-PCR. Consistently,

the expression of HO-1 in the 8-MOP group was significantly increased at 24 h compared with the vehicle group ($p<0.05$) (Figure 4A and 4C). In summary, these findings demonstrated that 8-MOP mainly protected the blood brain barrier *via* the Nrf-2/HO-1 pathway.

Discussion

Cerebrovascular disease has become the main cause of disability and death in the world. Although significant progress has been made in the pathophysiology of cerebral infarction, the improved treatment of cerebral infarction is still the focus of scientific research[21]. Therefore, it is of great importance to explore new therapeutic targets for cerebral infarction and provide new therapeutic strategies for cerebral infarction treatment.

After constructing the MCAO model, we detected the brain water content and evaluated the neurological function scores by the modified longa scoring method[20] in experimental rats. After the 8-MOP intervention, the neurological deficit

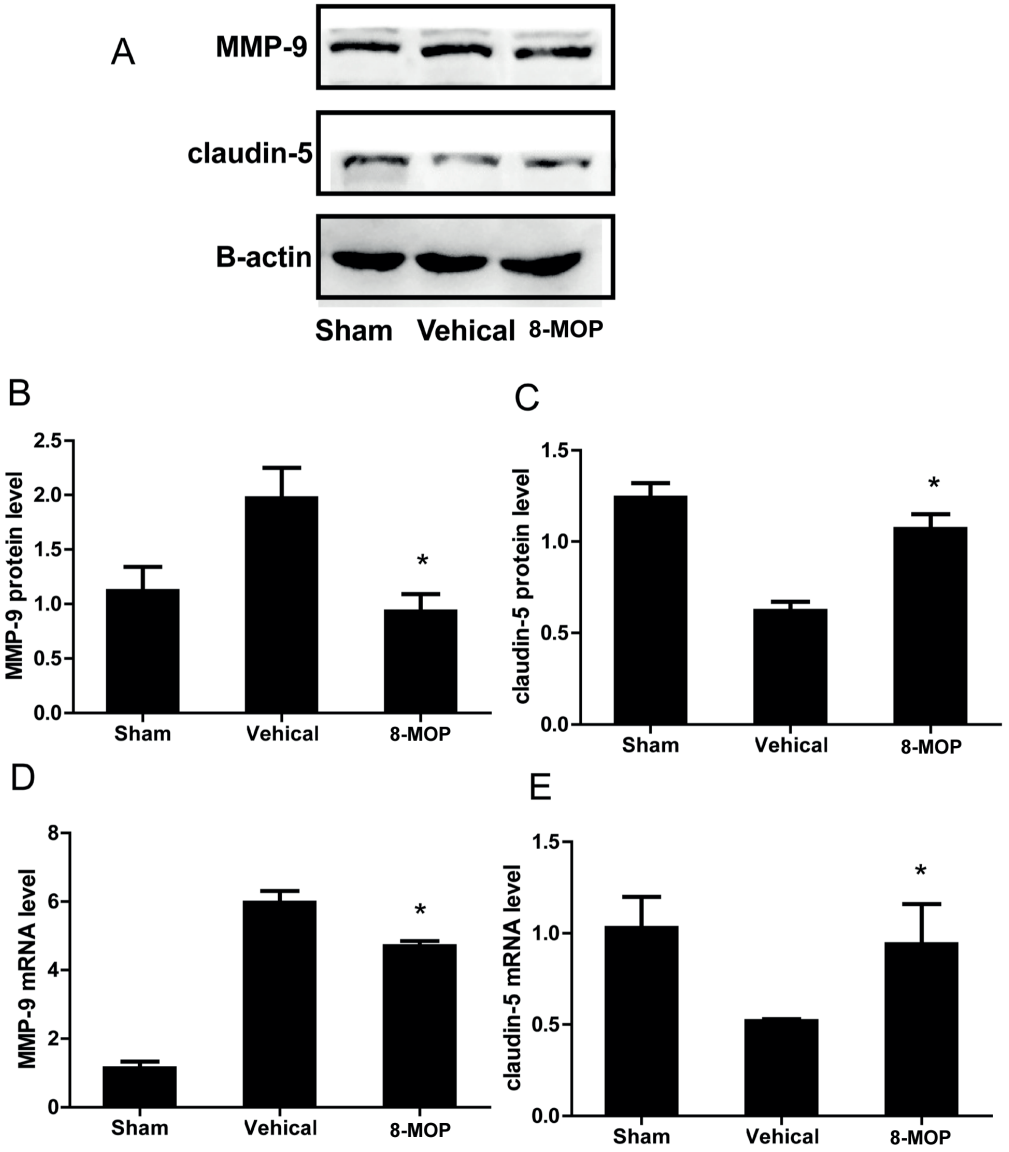

Figure 2. Effect of 8-MOP on the expression of MMP-9 and claudin-5. ***A,*** The expression of MMP-9 and claudin-5 in different groups were detected by Western blotting. ***B, C,*** The expression of MMP-9 and claudin-5 protein in the 8-MOP treatment group was significantly changed compared with the vehicle group ($p<0.05$). ***D, E,*** Histograms showed mRNA levels of MMP-9 and claudin-5 in different groups of brain tissue (*$p<0.05$ compared with vehicle group).

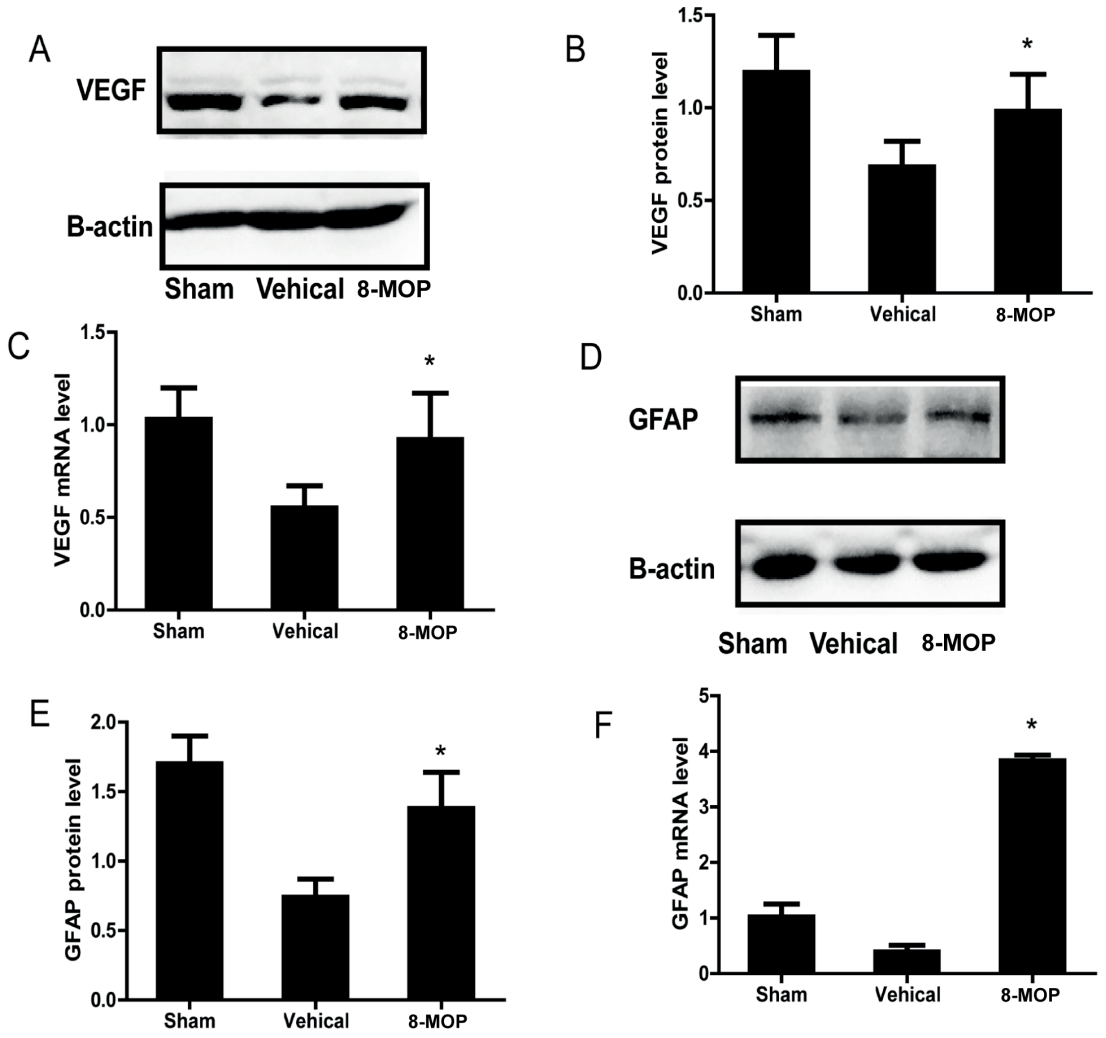

Figure 3. Effect of 8-MOP on the expression of endothelial growth factor VEGF. ***A-B,*** The expression of VEGF in different groups was detected by Western blotting. Compared with vehicle group, the expression of VEGF protein in the 8-MOP group was significantly increased ($p<0.05$). ***C,*** The histogram showed the mRNA levels of VEGF in brain tissue of different groups. Compared with vehicle group, the mRNA level of VEGF in 8-MOP group was significantly increased ($p<0.05$). ***D-E,*** The expression of astrocyte activation marker GFAP was detected by Western blotting in different groups. Compared with vehicle group, the expression of GFAP protein in the 8-MOP treatment group was significantly increased ($p<0.05$). ***F,*** Histograms showed the mRNA levels of GFAP in brain tissue of different groups. Compared with vehicle group, the mRNA level of GFAP in 8-MOP group was significantly increased. *$p<0.05$ compared with vehicle group.

scores of mice were decreased compared with the vehicle group, suggesting that 8-MOP could improve neurological deficits in mice with cerebral infarction and exerted a protective effect on the brain. The water content of brain tissues was detected by the standard dry weight method so as to determine the degree of cerebral edema in mice with cerebral infarction. Compared with the vehicle group, the water content of brain tissues in mice treated with 8-MOP was significantly reduced at 24 h, which further indicated that 8-MOP may play a protective role in cerebral ischemic brain tissue. In addition, high- and mid-dose of 8-MOP could both significantly reduce neurological deficit scores and brain edema in mice compared with the vehicle group. However, low-dose of 8-MOP had no significant effect on the cerebral infraction, suggesting that 8-MOP exerted brain protection in a dose-dependent manner.

Tight junction protein dysfunction is an important aspect of the pathogenesis of cerebral infarction. In MCAO rats, tight junction proteins inclu-

ding claudin-5, occludin, and zonula occludens1 (ZO-1) are downregulated while the blood brain barrier permeability is increased[22]. Studies have shown that matrix metalloproteinases (MMPs) play a special part in the pathophysiological mechanism of the blood-brain barrier dysfunction, which can degrade the tight junction proteins including claudin-5[23]. As the brain damage continues to worsen, matrix metalloproteinases are activated, which further aggravates the tissue damage, cerebral edema, and micro bleeds in the brain tissue and leads to cell death. Previous studies have shown that MMP-9 is correlated to the cerebral infarct area[24]. Our study confirmed that the permeability of blood-brain barrier in ischemic brain tissue increased and its integrity was destroyed 24 hours after cerebral infarction establishment. Meanwhile, 8-MOP treatment significantly reduced the permeability of blood-brain barrier and maintained its integrity, thereby reducing brain tissue damage. In detail, 8-MOP treatment markedly decreased the expression of MMP-9 in ischemic brain tissue, suggesting that 8-MOP could effectively inhibit MMP-9 activity. MMP-9 inhibition could decrease the degradation of tight junction proteins and reduce the brain tissue damage and cell death caused by MMP-9 activation, thereby reducing cerebral edema and the area of cerebral infarction. Moreover, 8-MOP treatment increased the expression of claudin-5, which further increased the tightness and stability of the blood-brain barrier.

Endothelial cells, astrocytes, pericytes, and extracellular matrix constitute the skeleton of the blood brain barrier and provide support for its special structure. Vascular endothelial growth factor (VEGF) is a key mediator in promoting angiogenesis. VEGF binds to endothelial cell surface receptors and initiates multiple downstream pathways associated with angiogenesis[25]. Studies have shown that 3 h after cerebral infarction, an upregulation of VEGF was observed, which could last for 3 to 7 days[26]. Our study showed that 8-MOP could increase the level of VEGF in ischemic brain tissue, thereby initiating angiogenesis after infarction, promoting repair of endothelial cells, and maintaining vascular density in ischemic brain tissue. Astrocytes, along with the endothelial cells, participate in the formation of the blood brain barrier skeleton. 8-MOP promotes the recovery of cerebral infarction by modulating multiple functional mesenchymal stromal pathways in cells[27]. It has been shown that co-culture of astrocytes and endothelial cells could increase the expression of the transporter proteins and enhance the activity of metabolic enzymes comparing to the culture of endothelial cells alone. Besides, the production of tight junction proteins was increased and the blood brain barrier function was enhanced after co-culture[28]. Glial fibrillary acidic protein (GFAP) serves as the marker of astrocyte activation. It is also one of the components of the astrocyte skeleton, which helps to maintain the tightness of the astrocyte membrane. GFAP-deficient mice exhibited severe hypoxic intolerance

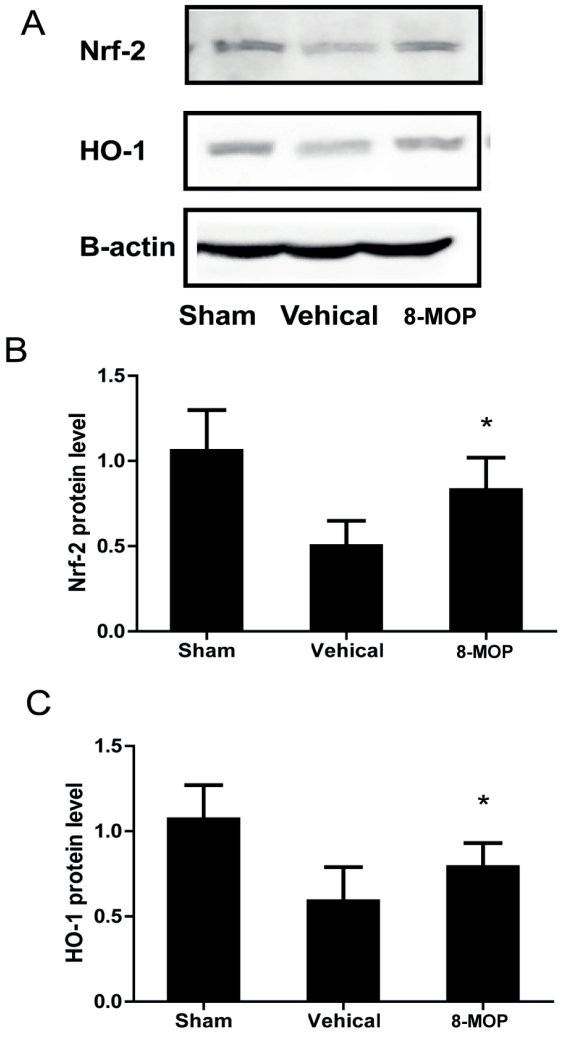

Figure 4. Effect of 8-MOP on the expression of Nrf-2 and HO-1. ***A, B,*** Western blot showed the expression of Nrf-2 in different groups. Compared with vehicle group, the expression of Nrf-2 protein in the 8-MOP group was significantly increased ($p<0.05$). ***C,*** Western blot showed the expression of HO-1 in different groups. Compared with vehicle group, the expression of HO-1 protein in the 8-MOP group was significantly increased. *$p<0.05$ compared with vehicle group.

after cerebral infarction modeling[29]. We showed that after 8-MOP treatment, the expression of GFAP increased, suggesting that 8-MOP could increase the tolerance to hypoxia in cerebral infarction mice and stabilize the astrocyte membrane skeleton as well as the blood brain barrier structure.

Heme oxygenases (HO) are ubiquitously present in mammalian microsomes and play an important role in endogenous antioxidant damage during cerebral ischemia and inflammation[30]. HO-1 is a free radical scavenger that is induced by oxidative stress, cytokines, ischemia-reperfusion, and other factors. The strong adaptability of HO-1 suggests that it plays an important role in resisting oxidative tissue damage and has a strong antioxidant capacity. Scholars[31-33] have shown that Nrf-2 is the most important transcription factor of HO-1. As a key factor in the cellular oxidative stress response, Nrf-2 is a member of the basic leucine zipper protein family with a highly conserved CNC (cap 'n' collar) structural region. Under physiological conditions, Nrf-2 repressively binds to Keap-1 at the amino terminus in the cytoplasm, and the Nrf2-Keap-1 complex mediates the expression of antioxidant enzyme genes. Under ischemic condition, where oxidative stress reaction occurs, Nrf-2 dissociates with Keap-1 and translocates into the nucleus, where it binds to the antioxidant response element (ARE) in the nucleus to promote the antioxidant enzyme gene expression and regulates many downstream protective factors[34]. The expression of Nrf-2 was found significantly up-regulated in brain tissue sensitive to ischemia and hypoxia. Activation of Nrf2 can increase the activity of various antioxidant enzymes and detoxification enzymes, and greatly increase the ability of cells to resist oxidation and detoxification. Besides, using Nrf-2 agonist sulforaphane to activate the Nrf-2 signaling pathway has been proved to improve the integrity and stability of the blood brain barrier in brain injury model[35]. In this investigation, we found that 8-MOP treatment could increase the protein expressions of Nrf-2 and HO-1, indicating that 8-MOP may play a part in brain protection by activating the Nrf-2/HO-1 signaling pathway to exert the blood-brain barrier protection.

The mouse cerebral infarction model was successfully established by using modified Longa method. As an ideal animal model for the study of cerebral infarction, this model has the characteristics of easy replication, high success rate, and stability. 8-MOP treatment could reduce the neurological deficit scores and cerebral edema in a dose-dependent manner, suggesting its role in brain protection in mice with cerebral infarction.

Conclusions

We showed that, 8-MOP could reduce the permeability of blood brain barrier and stabilize the blood brain structure, and also play a key role in initiating angiogenesis after infarction. Besides, 8-MOP could activate the Nrf-2/HO-1 signaling pathway, suggesting the protective effects of 8-MOP on blood-brain barrier *via* the Nrf-2/HO-1 pathway in mice model of cerebral infarction.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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