

Pelargonidin Ameliorates MCAO-Induced Cerebral Ischemia/Reperfusion Injury in Rats by Action on the Nrf2/HO-1 Pathway

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Research

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Abstract

Background: Morbidity and mortality remain high for ischemic stroke victims and at present there are no effective neuroprotective agents to improve the cure rate for these patients. In recent years, studies have shown that pelargonidin has many biological actions including anti-oxidant, anti-inflammatory and anti-thrombogenic effects. However, there are few reports about the treatment of cerebral ischemia with this agent.

Methods: The rat middle cerebral artery occlusion (MCAO) model was established to investigate the neuroprotective effect of pelargonidin on cerebral ischemia/reperfusion injury and to investigate its potential mechanism(s) of action. Magnetic resonance imaging and 2, 3, 5-triphenyltetrazolium chloride (TTC) staining were used to measure the volume of cerebral ischemia and modified neurological severity score (mNSS), the Morris water maze test to assess neurological functions, and ELISA to determine the levels of inflammatory factors in serum including TNF- α , TGF- β , IL-6 and IL-10 and oxidative factors i.e. MDA and SOD. The expression of Nrf2 and HO-1 protein in brain tissue was measured by immunofluorescence and western blot assays.

Results: The results showed that pelargonidin could effectively reduce the volume of cerebral ischemia and improve the neurological function in MCAO rats, thereby enhancing memory and learning ability. With corresponding decreases in the expression of TNF- α , TGF- β , IL-6 and MDA, pelargonidin increased the level of IL-10 and SOD and promoted the expression of Nrf2 and HO-1 proteins in ischemic brain tissues.

Conclusions: Our data demonstrated that pelargonidin ameliorated neurological function deficits in MCAO rats and its potential mechanism of action was associated with overexpression of the Nrf2/HO-1 signaling pathway, which may provide a new approach to the treatment of cerebral ischemia or cerebral ischemia/reperfusion injury.

Background

Stroke is a group of diseases associated with sudden rupture of cerebral vessels or brain tissue injury caused by blockage of blood flow to the brain and is characterized by high morbidity, mortality and disability rates. According to epidemiological studies, approximately 80.1 million people suffer from stroke worldwide, of which 41.1 million are female and 39 million male; in 2016, 13.7 million patients were newly diagnosed with stroke [1]. Stroke can be classified into two types: ischemic and hemorrhagic, with about 60–80% of strokes being ischemic in nature [2]. Although recombinant tissue-type plasminogen activator (r-tPA) is currently the most effective way to restore blood supply in ischemic stroke, only about 3–5% of ischemic stroke patients are effectively treated due to a narrow time window for r-tPA treatment [3, 4]. Patients with ischemic stroke may benefit from neuroprotective agents in the subacute phase or in the late stage of blood flow restoration [5]. Therefore, it is important to find effective neuroprotective agents that can successfully treat ischemic stroke.

Nuclear factor-E2-related factor 2 (Nrf2), a member of the leucine zipper family of transcription factors, plays an important role in reducing oxidative stress as an endogenous factor in brain tissue [6, 7]. In response to cellular oxidative damage, activated Nrf2 translocates into nuclei and binds to antioxidant response elements, thus regulating the expression of the downstream antioxidant enzyme heme oxygenase 1 (HO-1). HO-1 and its enzymatic products possess anti-oxidant, anti-inflammatory, anti-apoptotic and vasodilation actions, with concomitant improvement in the tissue microcirculation [8, 9]. Recently, it has been shown that Nrf2 activation attenuated oxidative damage induced by cerebral ischemic injury and that HO-1-deficient mice exhibit more severe brain injury compared with wild-type mice [10, 11]. As a result, the Nrf2/HO-1 pathway may be considered as a potential target for neuroprotective therapy in ischemic brain injury.

Anthocyanidins have potent anti-oxidant and anti-inflammatory effects [12]. As an anthocyanidin, pelargonidin (chemical formula shown in Fig. 1A) is widely distributed in vegetables and fruits, e.g. carrots, berries, blueberries, strawberries and pomegranates [13, 14]. Pelargonidin has been shown in several studies to have anti-oxidant [15], anti-inflammatory [16], anti-thrombotic [17] and anti-diabetic [18] activity among other functions. In addition, pelargonidin can ameliorate memory impairment in a rat model of Alzheimer's disease by inhibiting glial activation and oxidative stress [19]. However, the potential biological activities of pelargonidin as an anti-oxidant and anti-inflammatory factor in cerebral ischemia/reperfusion injury and its mechanism of action remain unclear.

In the present study, the rat MCAO model was used to investigate the neuroprotective effect of pelargonidin on cerebral ischemia/reperfusion injury and its potential mechanism(s) of action. Our study showed that pelargonidin could effectively reduce the infarct area in rats, improve their neurological functions, significantly reduce the level of inflammatory and oxidative factors in brain tissue after cerebral ischemia/reperfusion, and promote the repair of neuronal cells. The neuroprotective effect of pelargonidin on cerebral ischemia/reperfusion injury was associated with overexpression of the Nrf2/HO-1 pathway.

Materials And Methods

Animals

- Male Sprague-Dawley (SD) rats (220–260 g) were purchased from Shanghai Alac Laboratory Animal CO.LTD (Shanghai, China). License No.: SCXK (Shanghai) 2017-0005, Certificate No.: 20170005008495. All rats were fed a standard rodent diet and sterilized secondary ultrapure water *ad libitum*, housed at 22–25 °C, humidity 40–70% in a 12 h light-dark cycle. The animals were left to acclimatize for 7 days.

Group assignment and drug administration

60 SD rats were randomly divided into 4 groups (n = 15): Sham, MCAO, MCAO + Pel 10 mg/kg and MCAO + Pel 20 mg/kg. Pelargonidin (Chengdu Herbpurify CO., LTD, EINECS No.: 205-127-7, purity \geq 98%, Chengdu, China) was dissolved in sterile distilled water. Rats in the experimental groups were orally administered 10 mg/kg or 20 mg/kg of pelargonidin per day, while the Sham and MCAO groups were given the same volume of normal saline daily, both for 7 days (Fig. 1). The Animal Ethics Committee of Hainan Medical University approved all the animal experimental protocols.

MCAO model

The rat model of focal cerebral ischemia/reperfusion injury was prepared using the suture-occluded method developed by Longa. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (Sigma-Aldrich, CA, US, 300 mg/kg) and then fixed in the supine position. The neck was shaved and disinfected for routine skin preparation. A midline cervical incision was made to dissect the right common carotid, external and internal carotid arteries, followed by ligation of the external carotid artery and also its distal end. The proximal ends of the common carotid and internal carotid arteries were temporarily clipped. A small incision was made in the external carotid artery adjacent to the common carotid bifurcation and a silicon-coated suture inserted. The clip over the internal carotid artery was removed and the suture was gently inserted into the internal carotid artery through the external carotid artery until the origin of the middle cerebral artery was occluded; the length was typically 18–20 mm. The suture was tightened and the clip over the common carotid artery was removed, followed by cervical skin closure. After the ischemia status was maintained for 2 h, the suture was removed, allowing reperfusion of the blood supply. In the Sham group, only the internal carotid artery was dissected without any other procedure. After surgery, 100,000 units of penicillin sodium (Sigma-Aldrich, CA, US) were injected intramuscularly for 3 consecutive days to prevent infection.

MRI scanning

After treatment, the rats were examined using MRI scanning (GE Discovery MR750W 3.0T Superconducting Magnetic Resonance Imaging System) using a 3T experimental coil (5 cm in aperture). The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg) and fixed in the supine position, with the head placed through the coil centrally. A T2-weighted MRI scan (T2WI) and coronal diffusion-weighted imaging (DWI) was performed. An echo planar imaging (EPI) sequence was obtained for DWI with TR = 350 ms, TE = 50 ms, b value = 1,000 s/mm², slice thickness = 3 mm and slice gap = 0.2 mm. The images were processed by Functool software and the largest slice of ischemic lesions was selected for analysis. ROI with an area of 2 mm² was placed in the lesion center and contralateral mirror location. The relative apparent diffusion coefficient (rADC) and index ADC (eADC) were measured. rADC and reADC, (rADC = ipsilateral ADC value/homologous contralateral ADC value, reADC = ipsilateral eADC value/homologous contralateral eADC value) were calculated.

Neurological function tests(mNSS)

On day 2 after the end of treatment, the mNSS was used to evaluate the neurological functions of MCAO rats in each group; mNSS includes motor, sensory, reflex and balance functions, with a total score of 18.

The function was considered normal when the mNSS score was 0, and a higher mean mNSS score indicated a higher severity of neurological impairment [20]. The scoring details are shown in Supplementary Table 1.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), after which the thoracic cavity and right auricular appendix were opened. They were then transcardially perfused with PBS and then whole brain removed and sectioned into 5 slices. The sections were incubated in 2% TTC (Sigma-Aldrich, CA, US) at 37 °C for 30 min, fixed in 4% formaldehyde for 24 h and then photographed. Additionally, the infarct size was calculated.

Morris water maze (MWM) testing

The MWM test was performed to assess the spatial learning and memory of the rats. A round pool (120 cm diameter, 50 cm height, 30 cm depth, water temperature 22 ± 1 °C) was placed in an independent light-protected laboratory house and divided into four quadrants (E: East, S: South, W: West, N: North). Each rat was trained twice daily before the MWM at 120 s/dose for 3 days on end of treatment. On days 4, 6, 8 and 10, the place navigation test was performed. The platform was placed in any quadrant 2 cm underwater. The adjacent and opposite quadrants of the platform were selected as water entry points. The latency and times of crossing the platform were measured during a 120 s test. The assay was performed according to the instructions of the instrument supplier (SuperMaze Morris Water Maze Experimental Analysis System, Shanghai XinRuan Information Technology Co., Ltd.).

ELISA

Blood was collected from the abdominal inferior vena cava. After standing at room temperature for 2 h, it was centrifuged at 3,000 rpm for 10 min at 4 °C to separate the serum. The level of TNF- α , TGF- β , IL-6, IL-10, MDA and SOD was measured as using an ELISA kit (R & D Systems, Minneapolis, US).

Immunofluorescence

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg) and decapitated to facilitate removal of their brains. Brain tissues were fixed in 4% paraformaldehyde for 72 h and thereafter were embedded in paraffin and sectioned (4 μ m slices). The sections were dehydrated with gradient alcohol and washed with PBS 3 times. They were blocked with 10% fetal bovine serum (Gibco Life Technologies, NY, US) for 2 h and then incubated with anti-Nrf2 antibody and anti-HO-1 antibody (1:100, Abcam, Cambridge, MA, US) at 4 °C overnight, followed by washing with PBS three times. Fluorescence (red light) conjugated secondary antibody IgG (1:200, MultiSciences, Shanghai, China) was added and incubated for 2 h at room temperature, followed by washing with PBS three times. The slides were counterstained with DAPI (Beyotime Biotechnology, Shanghai, China) for 10 min and photographed under a fluorescence microscope.

Western blot analysis

The ischemic cortex tissues were isolated and homogenized using RAPI lysate (Beyotime Biotechnology, Shanghai, China) until no obvious lump was observed. Tissues were then centrifuged at 14,000 rpm for 30 min at 4 °C to collect the supernatant containing the total protein. The protein concentration of samples was determined using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). The protein samples were electrophoresed using 10% SDS-PAGE after they were transferred onto PVDF membranes (Millipore, MA, US), blocked with 5% skimmed milk for 2 h, and washed three times in TBST buffered saline. Next, they were incubated with Nrf2 and HO-1 (1:1000, Abcam, Cambridge, MA, US) monoclonal antibodies at 4 °C overnight. Subsequently, the membranes were washed 3 times with TBST buffered saline, incubated with anti-IgG antibody (1:2000, MultiSciences, Shanghai, China) at room temperature for 1 h, and washed with TBST buffered saline 3 times. The ECL Chemiluminescent kit (Beyotime Biotechnology, Shanghai, China) was used in a dark room for gel-imaging acquisition and analysis. Quantity One software was used to analyze the corresponding grayscale values of each band.

Data analysis

Data from 5 rats were collected separately in each experiment for statistical analysis. For each experiment, the data was expressed as the mean \pm S.E.M. and was statistically analyzed using GraphPad Prism 6.0 software. Statistical differences between data were assessed using one-way ANOVA followed by the Newman-Keuls test. A P -value < 0.05 was considered to be a statistically significant finding.

Results

Pelargonidin reduced the cerebral ischemic area in MCAO rats

MRI and TTC staining were used to measure the extent of cerebral infarction in MCAO rats treated with pelargonidin. Figure 2B, C shows that TTC staining failed to reveal any infarct areas in the sham group but there was an obvious white infarct area in the MCAO group (23.30 ± 4.10). Rats in the pelargonidin 10 mg/kg (17.29 ± 3.52) and 20 mg/kg (14.24 ± 3.04) groups had significantly reduced infarct areas compared to rats in the MCAO group ($P < 0.05$). On MRI, the T2WI sequence revealed no abnormalities in the bilateral cerebral hemispheres of the sham rats, but an abnormal signal intensity in the MCAO group. Rats in both the pelargonidin 10 mg/kg and 20 mg/kg groups had significantly fewer infarct lesions compared to rats in the MCAO group, which exhibited enlarged infarct lesions in brain tissue. The rADC values for both the MCAO and pelargonidin-treated rats were significantly lower than for rats in the sham group. Rats in the pelargonidin 10 mg/kg and 20 mg/kg groups had rADC values that were higher than those in the MCAO group. Although rats in both the MCAO and pelargonidin-treatment groups had higher reADC values than rats in the sham group, the reADC values in both the pelargonidin 10 mg/kg group and pelargonidin 20 mg/kg group were lower than those in the MCAO group (Fig. 3A-C).

- Pelargonidin improved neurological functions in MCAO rats

We used mNSS to assess the recovery of neurological functions in the MCAO rats (a higher mNSS score indicated less recovery). As shown in Fig. 3D, the mean mNSS score in the sham group was 0, indicating normal neurological functions, while the mean mNSS scores in the MCAO group and pelargonidin-treatment groups were increased; however, both of the pelargonidin groups had a lower mean mNSS score than the MCAO group ($P < 0.05$). In addition, the MWM test was performed to analyze the spatial learning and memory capabilities of the rats. Figure 4A-C shows that a 4-day MWM test demonstrated that the latency to escape was shortened for all I/R rats. However, the latency to escape in both the pelargonidin 10 mg/kg and 20 mg/kg groups was significantly shorter than that in the MCAO for the different 4-day tests. Similarly, the rats in the pelargonidin 10 mg/kg and 20 mg/kg groups crossed the platform more frequently than rats in the MCAO group during different 4-day tests. These findings suggested that pelargonidin could partially improve the neurological functions of I/R rats, and thus enhance their memory and learning capabilities.

- Pelargonidin reduced the levels of inflammatory factors in the brains of MCAO rats and exerted anti-oxidative effects

The pathophysiological mechanisms underlying cerebral ischemia/reperfusion injury are very complex. Such injuries are commonly caused by the release of inflammatory factors and oxidative damage. Hence, ELISA was used to measure the levels of TGF- β , TNF- α , IL-6, IL-10, MDA and SOD in samples of rat blood serum. Figure 5A-F shows that the levels of TGF- β , TNF- α , IL-6, and MDA in the MCAO group were higher than in the sham group, as well as levels in both the pelargonidin 10 mg/kg and 20 mg/kg groups ($P < 0.05$). The serum levels of IL-10 and SOD in the sham group were higher than those in the MCAO group, as well as those in the pelargonidin 10 mg/kg and 20 mg/kg groups ($P < 0.05$). The above results suggested that pelargonidin attenuated inflammatory responses and the degree of oxidative damage to brain tissues in MCAO-induced rats.

- Pelargonidin exerted neuroprotective effects by activating the Nrf2/HO-1 pathway

To examine further the mechanisms by which pelargonidin protects against MCAO-induced ischemia/reperfusion injuries, immunofluorescence and western blot assays were carried out to analyze the expression of Nrf2 and HO-1 proteins (components of the Nrf2/HO-1 pathway) in the infarcted brain tissues of experimental rats. The results revealed that the levels of Nrf2 and HO-1 protein expression in the MCAO group were higher than in the sham group, but lower than in either the pelargonidin 10 mg/kg or 20 mg/kg groups ($P < 0.05$, Fig. 6, Fig. 7A-D). These findings indicated that the neuroprotection provided by pelargonidin was accompanied by overexpression of the Nrf2/HO-1 pathway.

Discussion

The MCAO-induced I/R rat model is frequently used for cerebral ischemia studies, because it reflects pathophysiological changes and alterations in gene expression that are similar to those associated with cerebral ischemia/reperfusion injuries in humans. A determination of infarct volume and a behavioral evaluation of neurological functions are important factors for measuring the effect of any treatment for

cerebral ischemia [21]. It has previously been shown that pelargonidin could significantly attenuate MDA and catalase activity in the hippocampus, decrease GFAP levels, and thereby improve memory and learning functions in a rat model of Alzheimer's disease [19]. In the present study, both MRI and TTC staining results revealed that pelargonidin could effectively reduce the infarct volume in rats with cerebral ischemia, and also enhance the performance of MCAO rats in terms of motor activity, sensory skills, reflexes and balance, and improve their memory and learning abilities. The above results also suggested that pelargonidin can improve the neurological functions of MCAO rats and may be a candidate for development as a neuroprotective agent for treatment of cerebral ischemia or reperfusion injuries.

When cerebral ischemia develops at the reperfusion stage, the compensation provided by pre-existing nerve cells in the ischemic and hypoxic brain tissues rises abruptly, and is accompanied by a dramatic increase in free radicals that mediate oxidative damage in the affected areas [22, 23]. As an end product of oxidation, MDA further aggravates any damage to cellular membranes. Because SOD is the leading scavenger of free radicals, the severity of oxidative damage in a cerebral ischemic area will depend on the balance between MDA and SOD levels [24, 25]. On the other hand, oxygen free radicals and other messengers that reside in the ischemic area will help to upregulate the production of adhesion molecules; this allows leukocytes to accumulate in microvessels and ultimately create a vascular obstruction. During this process, inflammatory factors such as TGF- β , TNF- α and IL-6 may also be produced. In addition to an increased infiltration of inflammatory cells, these factors also contribute to the extracellular release of numerous inflammatory mediators, which further aggravate damage to the ischemic area [26, 27]. Pelargonidin has been found to inhibit TGF β 1p-induced human umbilical vein endothelial cell hyperpermeability, the expression of cell adhesion molecules, as well as the adhesion and migration of leukocytes [28]. Pelargonidin also inhibits the LPS-induced inflammatory response, helps to reduce the expression of TNF- α , IL-6, NF- κ B and other factors, and decreases mortality due to LPS-induced endotoxemia in mice [29]. In the present study, we found that pelargonidin could decrease the levels of MDA (an oxidative factor), TNF- α , TGF- β and IL-6 in the serum of MCAO rats, and elevate the expression SOD (an anti-oxidative factor) and IL-10 (an anti-inflammatory factor). Our results suggest that pelargonidin can attenuate oxidative stress and inflammatory responses that occur in a MCAO-induced rat model of I/R.

The Nrf2/HO-1 pathway plays an important role in preventing oxidative stress *in vivo* [30]. Under physiological conditions, Nrf2 is predominantly retained in the cytoplasm by forming a complex with Keap 1. When exposed to external stress, the Nrf2 inducer reacts with Keap1 cysteine to release Nrf2 protein, which subsequently translocates to the cell nucleus, where it activates Nrf2 and downstream antioxidant enzymes [11]. HO-1 is an inducible rate-limiting enzyme for heme catabolism in the microsomal enzyme system. As an antioxidant enzyme targeting Nrf2, HO-1 is essential for preventing cerebral ischemic injuries, Parkinson's disease and other neurodegenerative disorders [31, 32]. Pelargonidin has been shown to reduce TPA-induced methylation of the *Nrf2* gene promoter region in murine epidermal JB6 cells and to enhance the expression of *HO-1* (a downstream target gene for Nrf2), and thereby helps to provide cytoprotection [33]. It has also been reported that pelargonidin upregulates the Keap1/Nrf2 signaling pathway and ameliorates citrinin-induced oxidative stress injuries in HepG2

cells [15]. In the present study, treatment with pelargonidin was used to increase the levels of Nrf2 and HO-1 protein expression in the brain tissues of rats with ischemic reperfusion injury.

Conclusions

The present study has demonstrated that pelargonidin attenuated oxidative stress and inflammatory damage in the cerebral ischemia/reperfusion tissues of rats, and thereby exerted neuroprotective effects. Our results also showed that the effects of pelargonidin were achieved by inducing overexpression of the Nrf2/HO-1 pathway. These findings provide a new perspective on the development of agents for protecting against cerebral ischemia and cerebral ischemia/

reperfusion injuries.

Abbreviations

MCAO

Middle cerebral artery occlusion; TTC:2, 3, 5-triphenyltetrazolium chloride; mNSS:Modified neurological severity score; MDA:Malondialdehyde; SOD:Superoxide Dismutase; Nrf2:Nuclear factor-E2-related factor 2; HO-1:Heme oxygenase-1; r-tPA:recombinant tissue-type plasminogen activator; MRI:Magnetic Resonance Imaging; MWM:Morris water maze.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

QN was responsible for the conception and design of the study. KF, CM, HZ, CL, FY, QN were responsible for acquisition and analysis of data; KF and CM were in charge of statistical analysis. KF, CM and QN drafted the manuscript; KF and QN revised and commented on the draft and all authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The Research work involve animals are received ethical approval from the Animal Ethics Committee of Hainan Medical University.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no conflict of interests.

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Figures

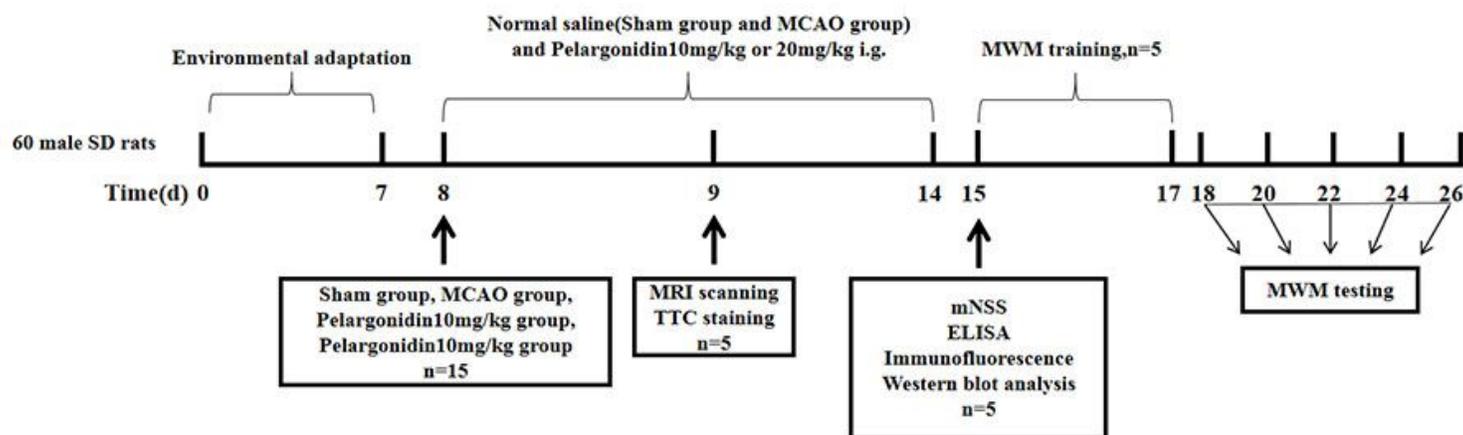


Figure 1

Flowchart of the MCAO experiments.

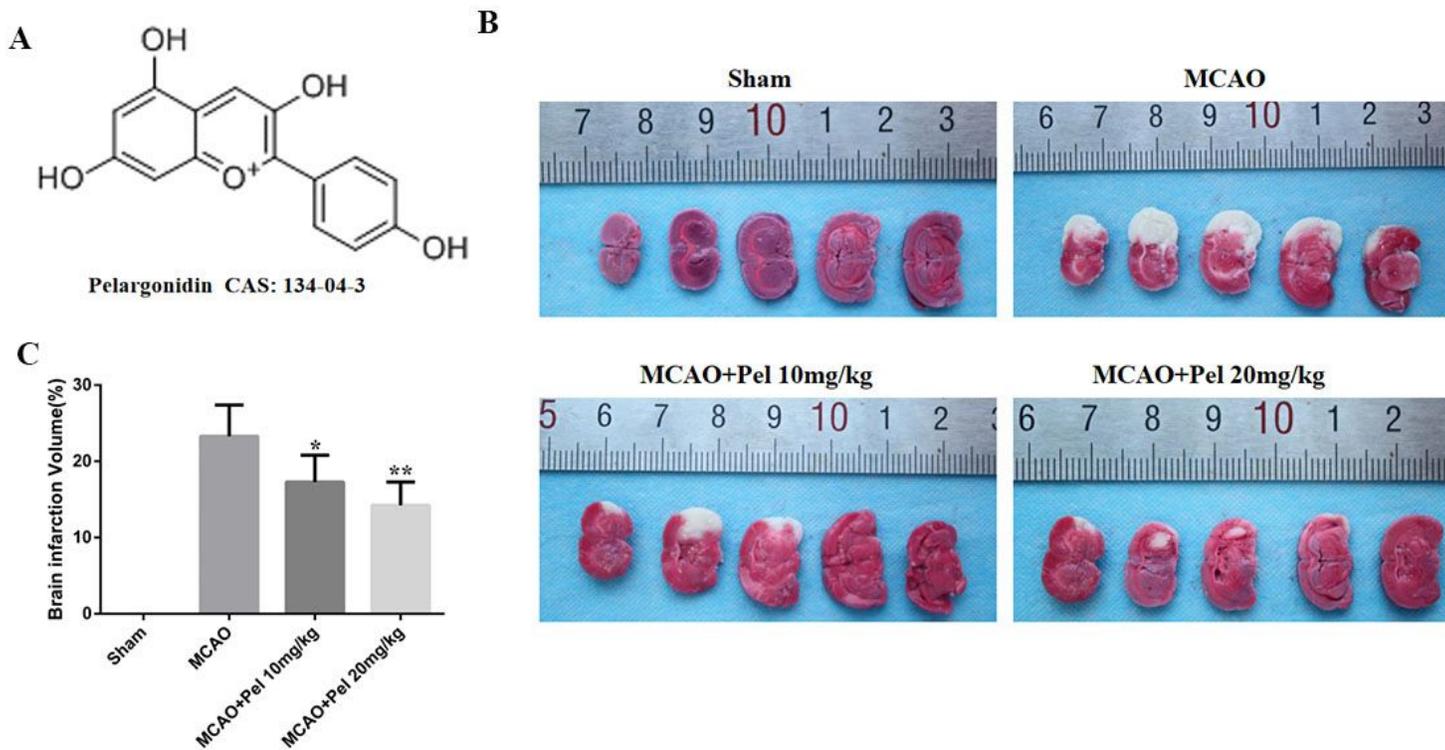


Figure 2

Cerebral infarction volume of rats in each group was compared. (A) Pelargonidin chemical formula and Chemical Abstracts Service (CAS) number. (B) TTC staining of typical brains of experimental rats. (C) Cerebral ischemic volume of experimental rats in each group (n=5). * P < 0.05, ** P < 0.01 compared with MCAO group.

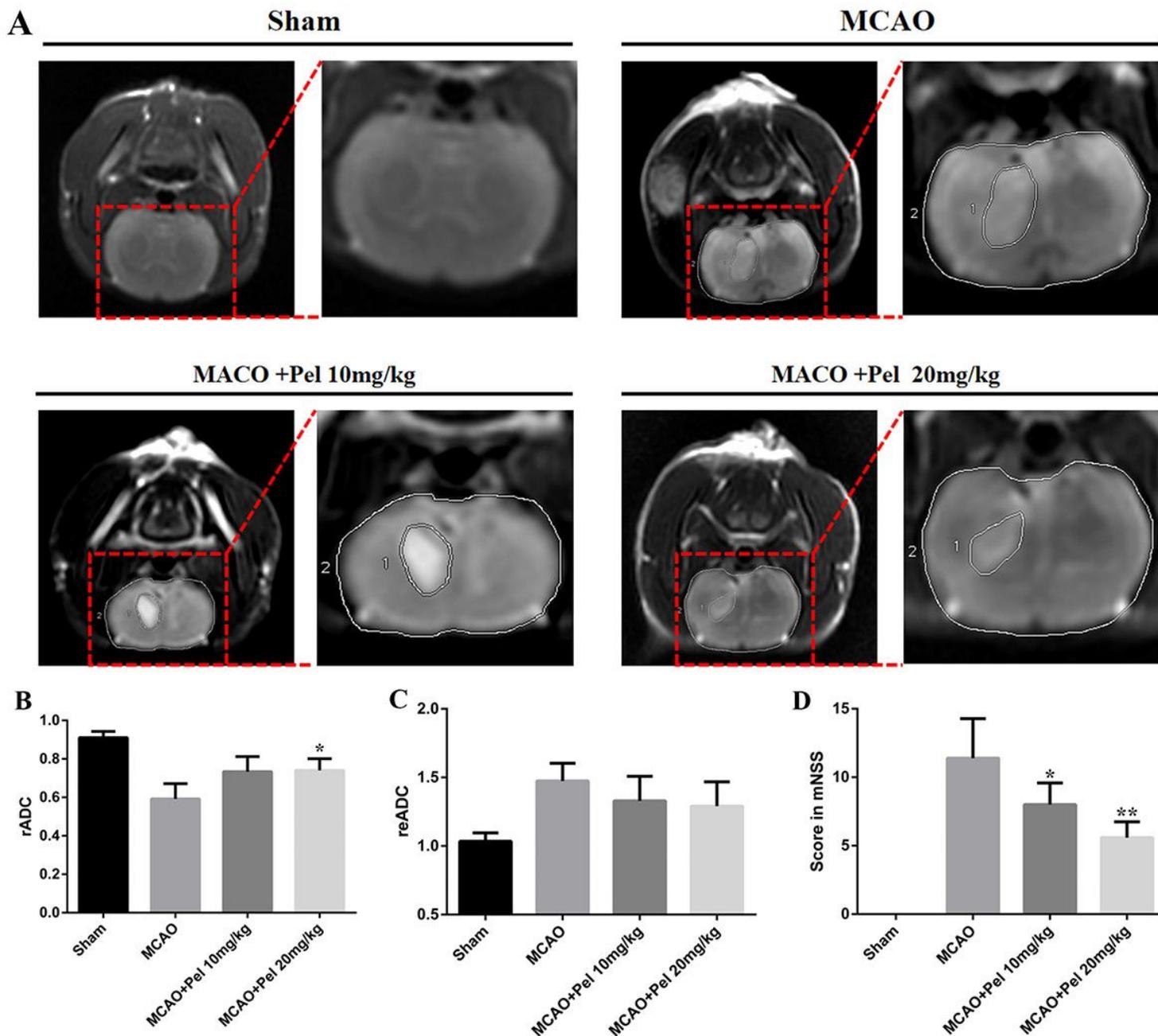


Figure 3

MRI detection of cerebral ischemia in each group of experimental rats. (A) Typical images of coronary angiography of T2WI scans of experimental rats in each group. (B-C) The rADC value and reADC value of experimental rats in each group (n=5). (D) The mNSS score was used to evaluate the effect of Pelargonidin on the neural function of rats in each experimental group (n=5). * P < 0.05, ** P < 0.01 compared with MCAO group.

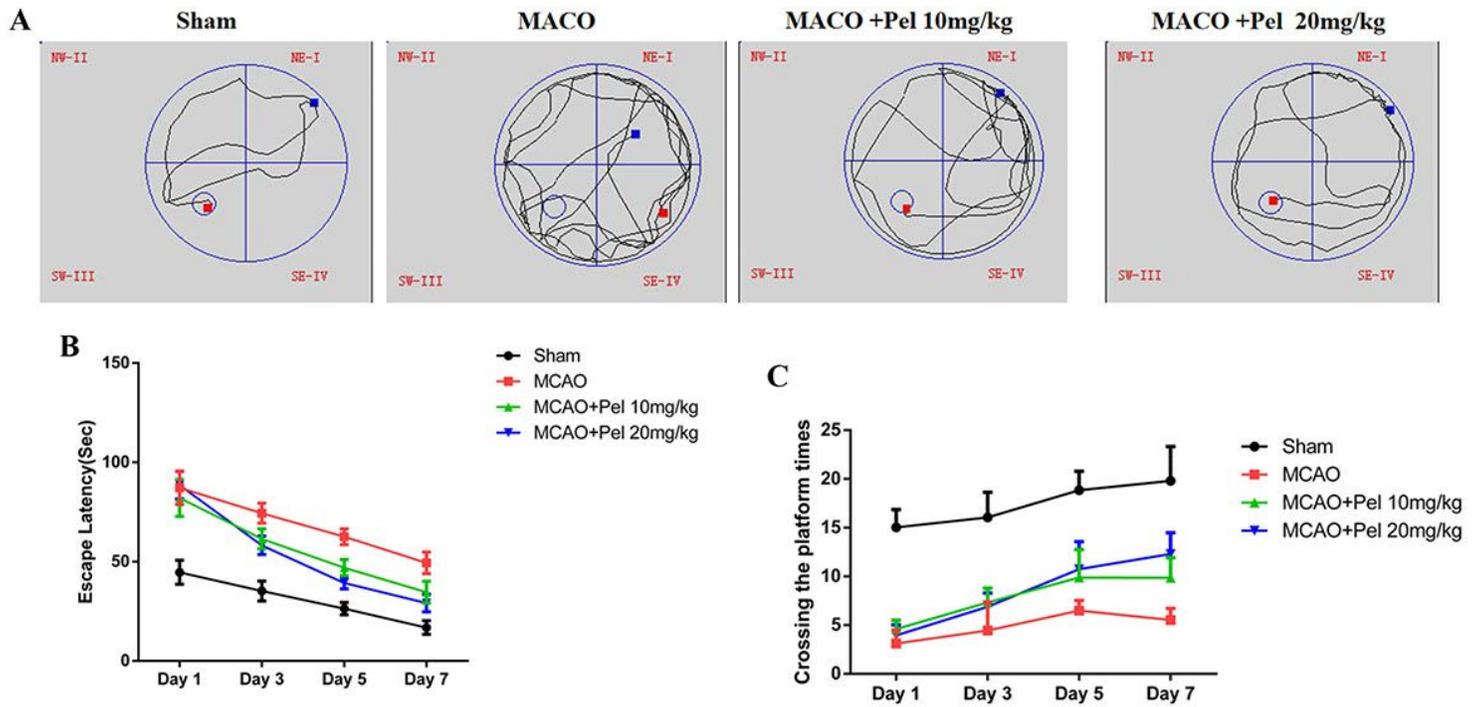


Figure 4

MWM was used to detect spatial learning and memory in rats. (A) Representative trajectories of rats in each experimental group (NE- \boxtimes : first quadrant, NW- \boxtimes : second quadrant, SW- \boxtimes : third quadrant, SE- \boxtimes : fourth quadrant; the small red square indicates the starting point and the small blue square indicates the end). (B) Total distance of each group within 2 minutes. (C) The number of times each group crosses the platform within 2 minutes.

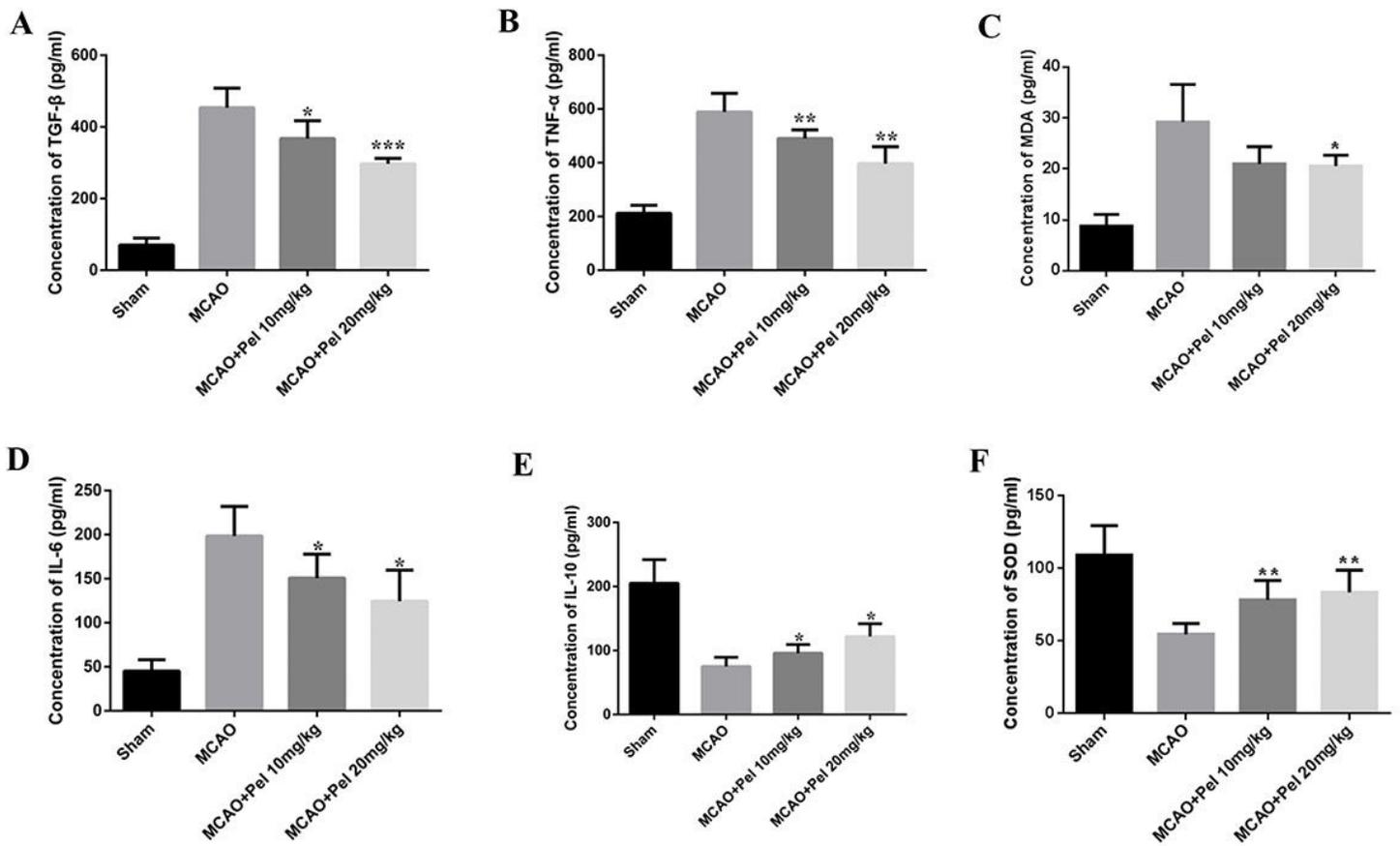


Figure 5

Serum levels of inflammatory factors (TNF-α, TGF-β, IL-6, IL-10) and oxidative factors (MDA, SOD) in rats of all experimental groups were determined by ELISA (n=5). * P < 0.05, ** P < 0.01, *** P < 0.001 compared with MCAO group.

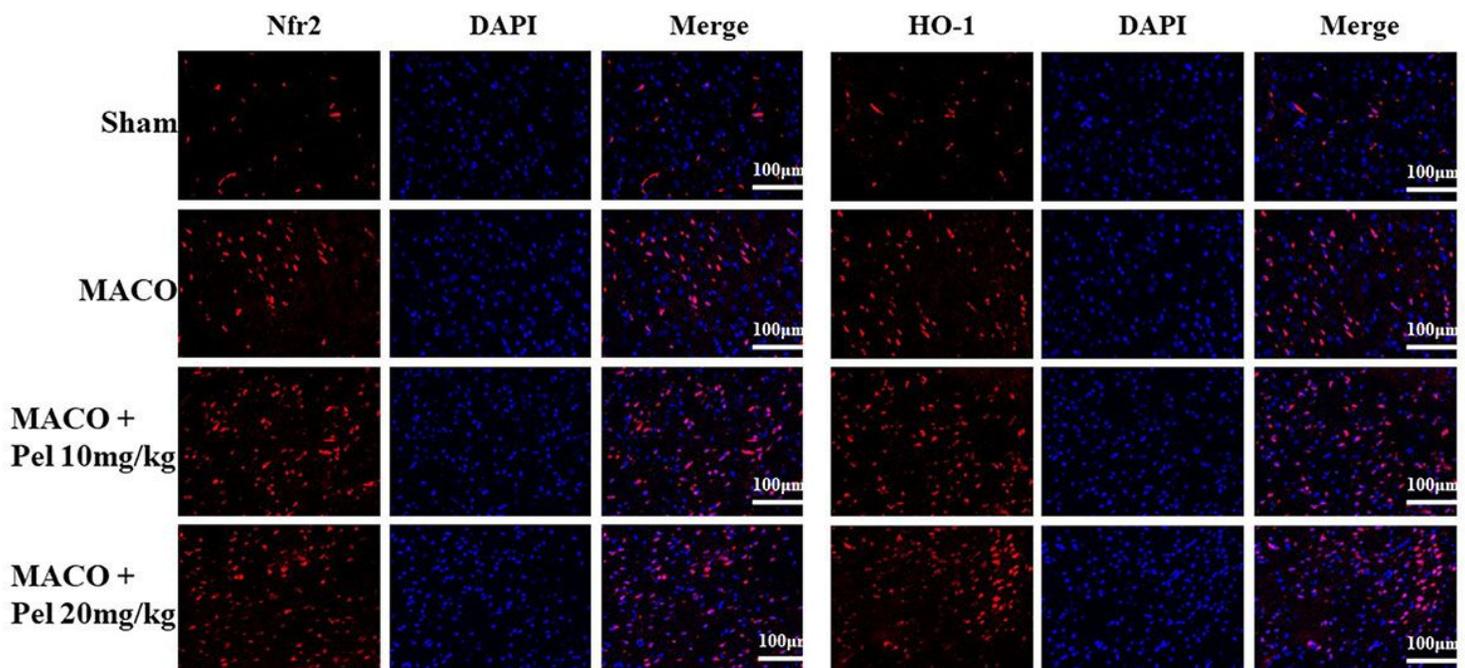


Figure 6

Immunofluorescence was used to observe the expression of Nrf2 and HO-1 in the brain tissue of rats in each group (scale bar = 100 μ m).

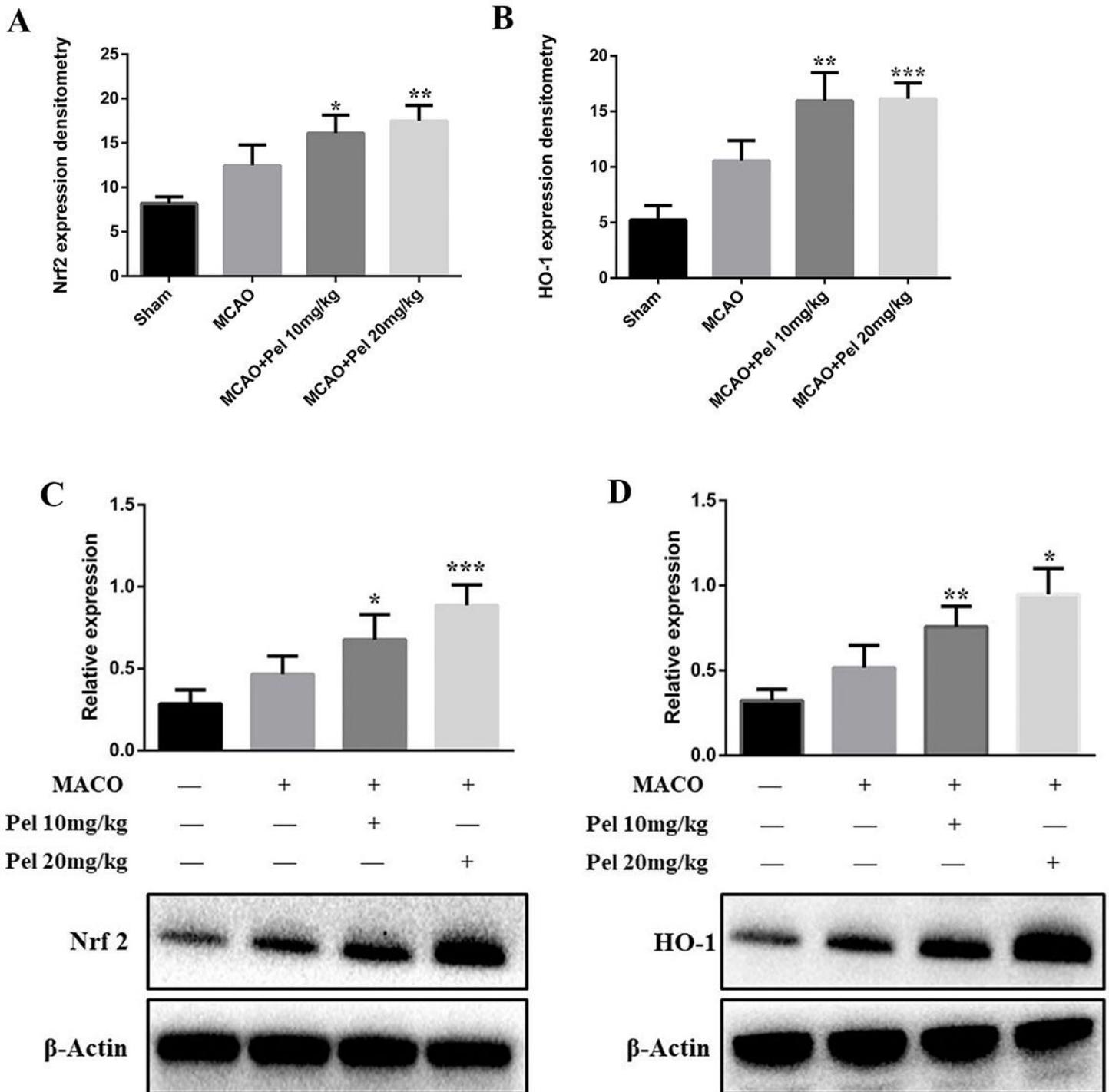


Figure 7

Expression levels of Nrf2 and HO-1 in rat brain tissue. (A-B) The relative expression of Nrf2 and HO-1 in rat brain tissue was detected by immunofluorescence (n=5). (C-D) The relative expressions of Nrf2 and

HO-1 in brain tissues of rats in each group were determined by Western Blot (n=5). * P < 0.05, ** P < 0.01, *** P < 0.001 compared with MCAO group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.doc](#)