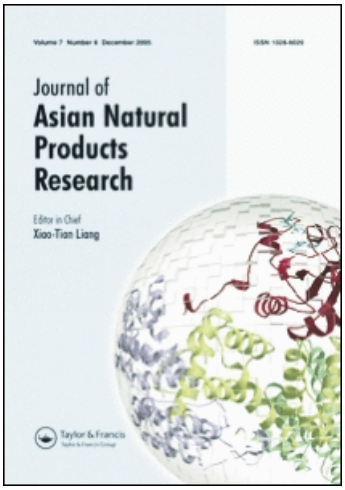

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Pinocembrin protects the neurovascular unit by reducing inflammation and extracellular proteolysis in MCAO rats

Mei Gao[a]; Shen-Yin Zhu[ab]; Chu-Bing Tan[a]; Bei Xu[a]; Wen-Cui Zhang[c]; Guan-Hua Du[a]
[a] Peking Union Medical College, Institute of Materia Medica and Chinese Academy of Medical Sciences, Beijing, China [b] Chongqing University of Medical Science, Chongqing, China [c] School of Life Science and Technology, Beijing Institute of Technology, Beijing, China

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ORIGINAL ARTICLE

Pinocembrin protects the neurovascular unit by reducing inflammation and extracellular proteolysis in MCAO rats

Mei Gao[a], Shen-Yin Zhu[ab], Chu-Bing Tan[a], Bei Xu[a], Wen-Cui Zhang[c] and Guan-Hua Du[a]*

[a]*Peking Union Medical College, Institute of Materia Medica and Chinese Academy of Medical Sciences, Beijing 100050, China;* [b]*Chongqing University of Medical Science, Chongqing 400016, China;* [c]*School of Life Science and Technology, Beijing Institute of Technology, Beijing 100081, China*

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The purpose of the present study was to examine the protective action and mechanisms of pinocembrin **(1)** on the neurovascular unit (NVU) in permanent cerebral ischemic rats. Focal cerebral ischemia was induced by occlusion of middle cerebral artery (MCAO) in rats. Compound **1** (3, 10, or 30 mg/kg) was intravenously injected at 0, 8, 16 h after MCAO. At 24 h of occlusion, **1** alleviated neuronal apoptosis, edema of astrocytic end-feet, and the deformation of endothelial cells and capillaries as revealed by the transmission electron microscopy study. To understand the mechanisms of action, the anti-inflammation effect of **1** was examined. Compound **1** reduced the expressions of tumor necrosis factor-α, interleukin-1β, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, inducible NO synthase and aquaporin-4; inhibited the activation of microglias and astrocytes; and downregulated the expression of matrix metalloproteinases (MMPs) in the ischemic brain. The ischemia-induced decreases in mRNA expressions of tight junction constituent proteins, occludin and ZO-1, were also inhibited by **1**. These results indicated that **1** can protect the rat brain against ischemia injury by inhibiting the inflammatory cascade, reducing the expression of MMP-9, and preventing the integrity of tight junction. This resulted in the protective action of **1** on the NVU.

Keywords: pinocembrin; stroke; neurovascular unit; inflammation; extracellular proteolysis

1. Introduction

Stroke is the third leading cause of death around the world and is associated with serious long-term physical and cognitive disabilities, especially in elderly patients [1]. Nearly 85% of strokes are ischemic, and neurons in the ischemic center (core) quickly undergo necrosis because of ATP depletion and ionic failure. The core is surrounded by a ring-like penumbra [2], which during a stroke is electrically silent but still has significant blood flow. Delayed oxygenation/reperfusion and apoptosis contribute to the vulnerability of this area to inflammation and free radical attack. The focal cerebral ischemia model using the middle cerebral artery occlusion (MCAO) technique is widely accepted as being closer to the clinical manifestations of stroke in humans. To date, there are no

*Corresponding author. Email: dugh@imm.ac.cn

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clinically effective therapies for brain damage because of ischemic stroke.

For a long time, the central nervous system (CNS) was considered to be 'immunologically privileged' due to shielding from access by immune cells by the blood–brain barrier (BBB) [3]. However, it is becoming apparent that leukocytes, as well as cytokines and chemokines, can cross the intact BBB and that there is a substantial crosstalk between peripheral and local immune components [4]. It is well known that stroke triggers a robust inflammatory reaction characterized by peripheral leukocyte influx into the cerebral parenchyma and activation of endogenous microglia. Ischemic cells, even ischemic neurons, secrete inflammatory cytokines that cause, among other things, adhesion molecule upregulation in the cerebral vasculature, which leads to peripheral leukocyte recruitment. Brain cells are also capable of secreting chemokines, leading to further inflammatory cell chemotaxis into the ischemic lesion. Once activated, inflammatory cells can release a variety of cytotoxic agents including cytokines, matrix metalloproteinases (MMPs), nitric oxide (NO), and more ROS. These substances can contribute to more cell damage as well as disruption of the BBB and extracellular matrix [5,6].

Preclinical studies suggest that interventions that are aimed at attenuating such inflammation reduce the progression of brain damage that occurs during cerebral ischemia. Therefore, there is a strong rationale for continuing to explore the efficacy of anti-inflammatory therapies in the treatment of acute and sub-chronic stages of cerebral ischemia.

Pinocembrin **(1)** (5,7-dihydroxyflavanone; Figure 1) is one of the most abundant flavonoids in propolis and has been reported to have multiple actions including anti-inflammatory, antioxidant, antimicrobial, anti-allergic, and antiviral activities [7–9]. Our previous study also found that **1** induced relaxation of rat aortic rings

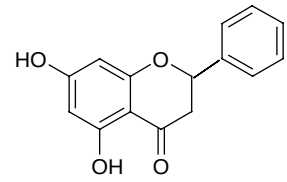

Figure 1. Chemical structure of **1**.

[10]; and that **1** could improve rat cognitive impairments induced by chronic cerebral hypoperfusion [11]. In the latest published materials, we demonstrated that **1** could reduce infarction, improve neurological disability, and prevent the integrity of the BBB in rats subjected to MCAO surgery [12]. Compound **1** could also protect rat brain against ischemia–reperfusion both *in vivo* and *in vitro* [13], and prevent glutamate-induced neuronal cell apoptosis [14].

In light of the specific anti-inflammatory action of **1** *in vitro,* and the deleterious inflammatory reaction during the onset and the late stages of ischemic injury, the purpose of the present study was to examine the action of **1** on permanent cerebral ischemia in rats, and investigate whether its anti-inflammatory effect contributed to the prevention in ischemia.

2. Results and discussion

2.1 Effect of 1 on the ultrastructure of the neurovascular unit

As shown in Figure 2, there was compact and continuous neuropil in brain slices of the sham-operated group. Vacuolized neuropil was observed in ischemic areas of model group rats, with severe swollen glial end-foot processes, apoptotic neurons, and swelling mitochondria. In **1**-treated groups, vacuolization of neuropil and astrocyte end-foot process edema were alleviated, and neurons were in good condition.

Normal cerebral microvessel and endothelial cells (ECs) were observed in the sham-operated group, showing patent

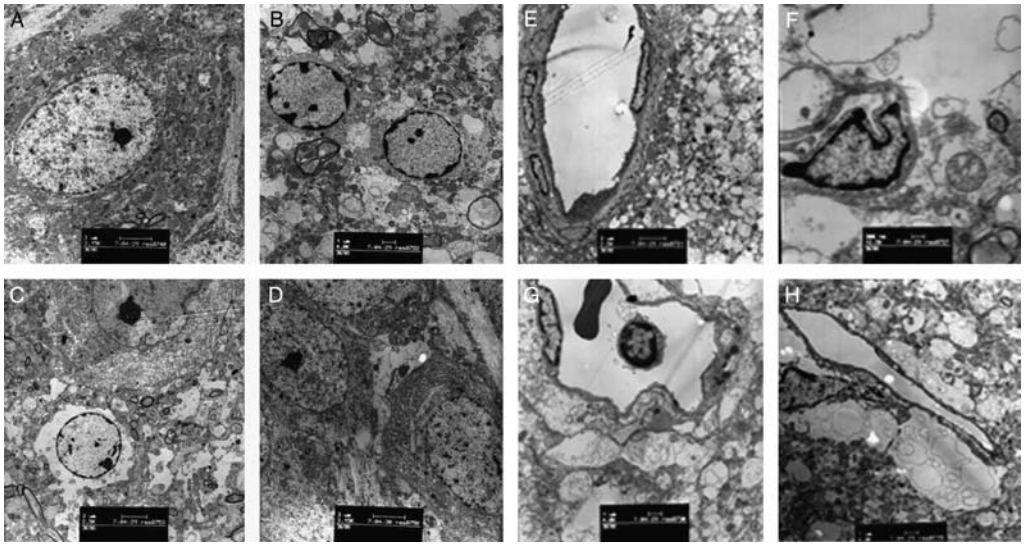

Figure 2. Effects of **1** on electron micrographs of neuropil (A–D), microvessels and ECs (E–H) 24 h after MCAO (× 3200). Representative images are from the sham-operated group (A and E); model group (B and F); **1** 3 mg/kg group (C and G); **1** 10 mg/kg (D and H). (A) Neurons (N) are in their normal condition. The adjacent neuropil is compact and continuous. (B) Severe swelling and vacuolization in neuropil; apoptotic neurons and swelling mitochondria (Mt) are observed. (C and D) Vacuolization of neuropil and astrocyte (As) end-foot process edema are alleviated; neurons are in good condition. (E) Capillaries with patent lumina (L) with some PC apposed to the capillary wall. (F) Marked edema of astrocytic (As) end-foot processes and enlargement of the EC nucleus and cytoplasm contribute to narrowing capillary lumen. Tight junction (TJ) is opened. Swelling mitochondria (Mt) is observed. (G and H) Edema of astrocytic end-foot and the deformation of ECs and capillaries are alleviated.

lumina with some pericytes (PC) apposed to the capillary wall (Figure 2(E)). At 24 h of occlusion, the capillary lumen was narrowed, nearly closed by marked edema of astrocytic end-foot processes. Enlargement of the EC nucleus and cytoplasm, tight junction opening and swelling mitochondria were also seen in ischemic areas of model group rats (Figure 2(F)). Treatment with **1** alleviated edema of astrocytic end-feet and the deformation of ECs and capillaries (Figure 2(G) and (H)).

2.2 Effect of 1 on the induction of inflammatory mediators at 24 h of MCAO

As shown in Figure 3, the cytokines, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) were highly expressed after 24 h of MCAO in the ipsilateral hemisphere (mainly penumbral cortex) of the ischemic brain, and these expressions were significantly reduced in **1**-treated animals. The secretions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) followed

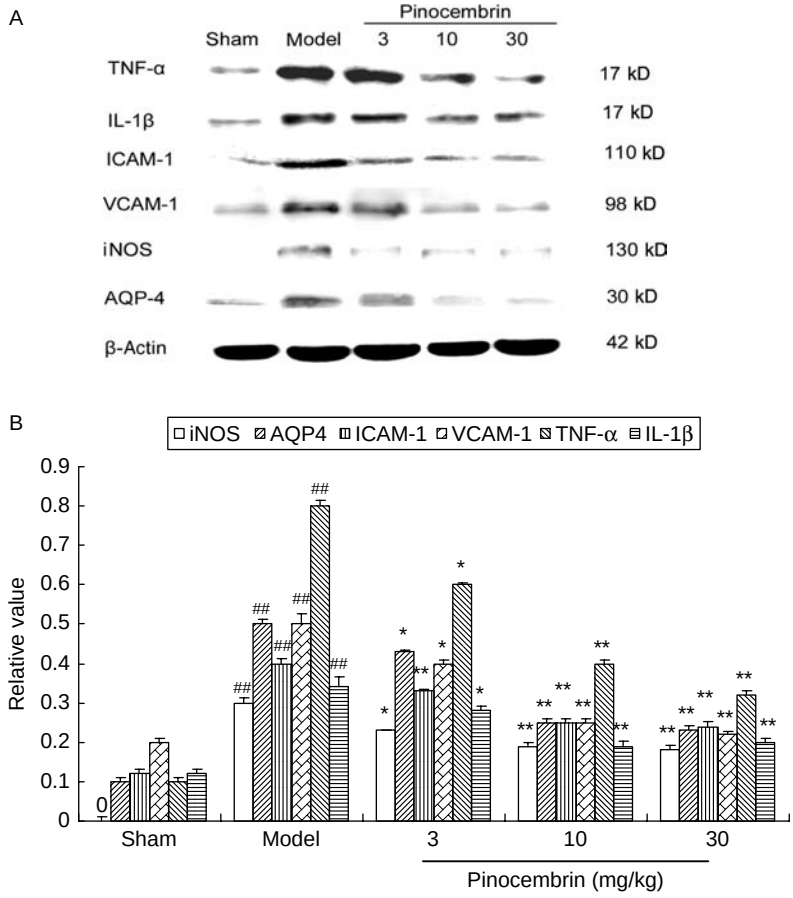

Figure 3. (A) Western blot of the rat brain at 24 h of MCAO. Representative Western blot from three different sets of experiments showed expressions of TNF-α, IL-1β, ICAM-1, VCAM-1, iNOS, and AQP-4 presented in the ipsilateral hemisphere. (B) Quantification of the bands (shown in (A)) determined by densitometric scanning and expressed as the ratio vs. β-actin. β-Actin was used as an internal control. $^{\#\#}P < 0.01$ vs. sham group, $*P < 0.05$ vs. model group, $**P < 0.01$ vs. model group.

a similar pattern for TNF-α and IL-1β, and treatment with **1** reduced ICAM-1 and VCAM-1 expressions drastically. The expression of inducible nitric oxide synthase (iNOS) in model group rats was elevated as well, and also decreased in **1**-treated groups. There is no detectable iNOS in the brain of sham-operated group animals. Aquaporin-4 (AQP-4) mainly expressing at the end-feet of astrocytes is closely related to brain swelling in cerebral ischemia. Our results showed that AQP-4 was also highly expressed at 24 h of ischemia, and reduced remarkably by **1** treatment. At this time point, the inhibition of TNF-α, IL-1β, ICAM-1, VCAM-1, iNOS, and AQP-4 at a high dose of **1** (30 mg/kg) exhibited as 60.5, 42.3, 40.4, 56.3, 40.1, and 54.1%, respectively (Figure 3).

2.3 Effect of 1 on activation of microglias, astrocytes, and the expression of MMP-9 at 24 h of MCAO

Inflammatory mediators, secreted by either infiltrating blood-borne cells or activated glia cells, enhance oxidative and nitrosative stresses and induce apoptotic cell death. OX42 and GFAP markers were used to detect activated microglias and astrocytes. There were significant specific staining for OX42 (Figure 4(A)–(D)) and GFAP (Figure 4(E)–(H)) in the penumbra region of the ipsilateral hemisphere in model group rats. Treatment with **1** (3 and 10 mg/kg) reduced the number of OX42-positive cells and GFAP-positive cells.

Because activation of MMP-9 and degradation of critical protein components of cerebral blood vessels have been demonstrated to be correlated with the development of hemorrhage and edema in ischemia [6], the expression of MMP-9 was also investigated. Compared with sham animals, the number of MMP-9-positive cells was significantly increased in the ischemic cortex after 24 h of ischemia in MCAO group rats, and treatment with **1** inhibited this increase (Figure 4(I)–(L)).

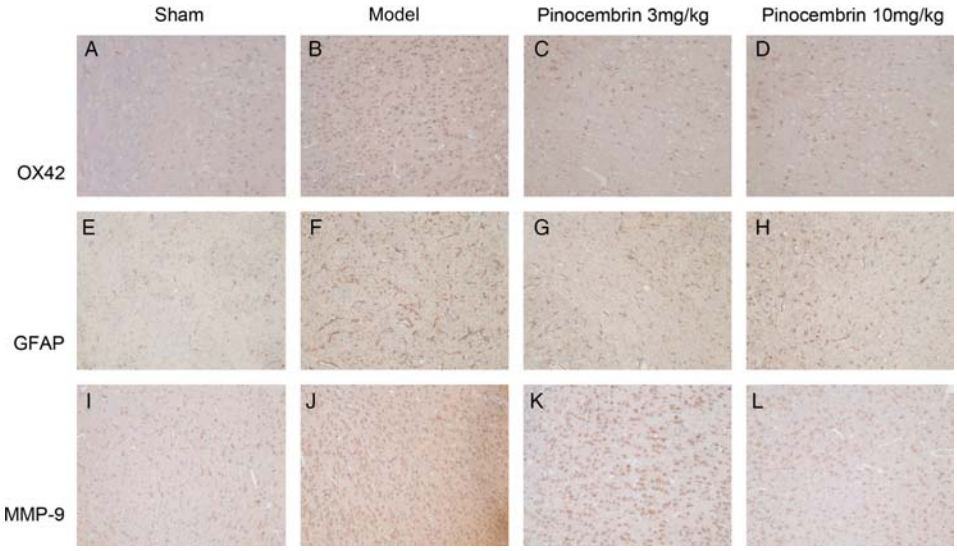

Figure 4. Immunohistological detection of the activation of microglials and astrocytes and the expression of MMP-9 in the ischemic cortex. Positive cells are brown stained. (A) Sham-operated group; (B) model group; (C) **1** 3 mg/kg-treated group; (D) **1** 10 mg/kg-treated group (× 100).

2.4 Effect of 1 on mRNA expressions of occludin and ZO-1

Occludin and ZO-1 are two main construction proteins of the endothelial tight junction. The mRNA expressions of occludin and ZO-1 in ischemic cortex areas were determined in our experiment. Semi-quantitative analysis of the RT-PCR products showed that the mRNA expressions of occludin and ZO-1 were significantly decreased by 85.1 and 49.0%, respectively, at 24 h of ischemia. Treatment with **1** (3 and 10 mg/kg) significantly inhibited the degradation of these proteins dose-dependently (Figure 5).

2.5 Effect of 1 on the expressions of iNOS, TNF-α, IL-1β, and AQP-4 in ischemic cortex 7d after ischemia

Seven days after occlusion, **1** improved the survival conditions of rats compared with the model group (data not shown). We reevaluated the anti-inflammatory effect of **1** at this time point by detecting the expressions of iNOS, TNF-α, and IL-1β, using immunohistology analysis. The results showed that the expressions of iNOS, TNF-α, and IL-1β in the model group are still kept at a relatively high level compared with the sham-operated group, and were reduced in the **1**-treated group. The continuous increased expression of AQP-4 was also detected in the model group, representing the brain swelling condition. Compound **1** reduced the expression of AQP-4 as well (Figure 6).

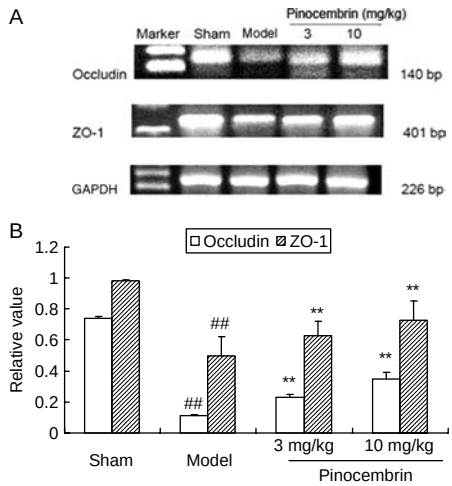

Figure 5. Effects of **1** on mRNA expressions of occludin and ZO-1. Quantification of the occludin and ZO-1 mRNA bands determined by densitometric scanning and expressed as the ratio vs. GAPDH. $^{\#\#}P < 0.01$ vs. sham group, $^{**}P < 0.01$ vs. model group.

Pinocembrin **(1)** is one of the most abundant flavonoids in propolis, and has been proven to have antimicrobial, antioxidant, anti-inflammatory, and vascular relaxation activities [7–9]. In the present study, we demonstrated for the first time that **1** can protect ultrastructure of the neurovascular unit (NVU) after focal cerebral ischemia in rats, by alleviating neuronal apoptosis, neuropil vacuolization, mitochondria swelling, astrocyte end-foot process edema, and deformation of ECs (Figure 2).

It is well established that cerebral ischemia is associated with the infiltration of inflammatory cells into the ischemic brain and activation of microglias and astrocytes [15,16]. Cytokines are important molecular signals in the inflammatory response to cerebral ischemia and can upregulate the expressions of adhesion molecules, such as ICAM-1, VCAM-1, selectins, and integrins, on ECs, leukocytes, and platelets. Adhesion receptors mediate the interaction between ECs and leukocytes, which results in the 'rolling' of leukocytes and in their adhesion to the endothelium of venules, followed by transmigration of these leukocytes into the brain parenchyma. On the other hand, inflammatory cells may contribute to neuronal damage by producing oxygen free radicals and other inflammation-related products that are toxic to neurons [15].

iNOS, a third isoform of NO synthase, is normally not present in most cells, but its expression is induced during development and in pathologic states, mostly

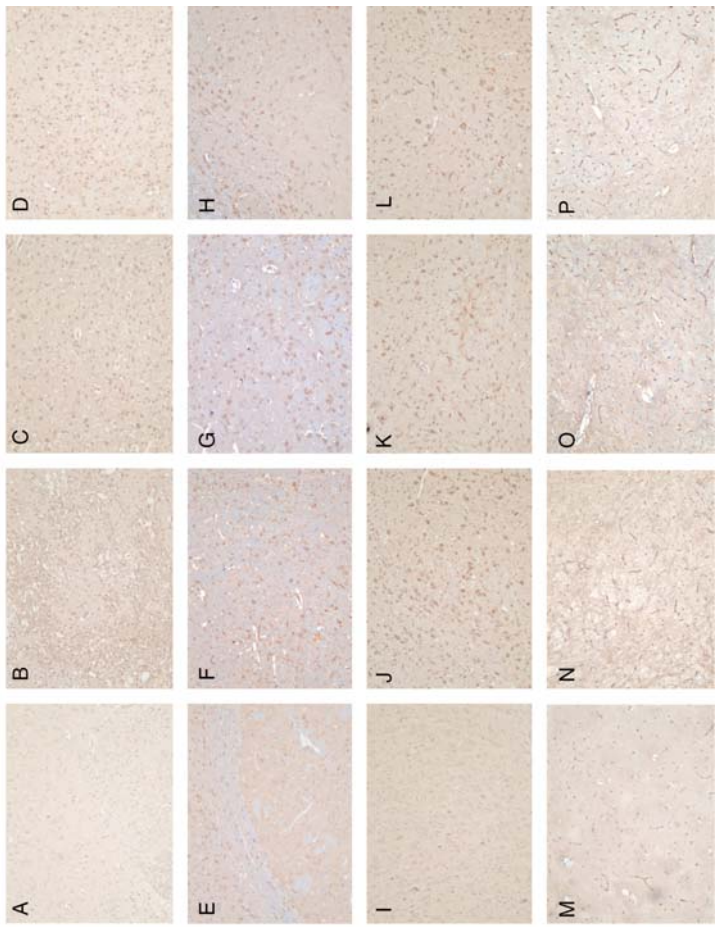

Figure 6. Immunohistological detection of the expression of iNOS, TNF-α, IL-1β, and AQP-4 in the ischemic cortex 7 days after ischemia. Positive cells are brown stained. (A) Sham-operated group; (B) model group; (C) **1** 3 mg/kg-treated group; (D) **1** 10 mg/kg-treated group (× 100).

associated with inflammation. iNOS generates toxic levels of NO, and is thought to contribute to the cytotoxicity induced by inflammation. In the brain, iNOS is induced in the setting of post-ischemic inflammation. It was reported that iNOS mRNA is upregulated and peaks at 12 ± 48 h after transient or permanent MCAO in rodents [17].

To be consistent with the above evidences, our results revealed that there were significant increases in expressions of TNF-α and IL-1β at 24 h in MCAO group rats compared with sham group rats. The secretions of ICAM-1 and VCAM-1 followed a similar pattern to those of TNF-α and IL-1β (Figure 3). We also detected a significant inducible expression in model group rats compared with under detectable level of iNOS in sham animals at 24 h after ischemia (Figure 3). Treatment with **1** dose-dependently reduced the expressions of these cytokines, adhesion molecules, and iNOS in the infarct area at both time points of 24 h and 7 d after MCAO (Figures 3 and 6). Compound **1** could also inhibit the activation of microglias and astrocytes after ischemia (Figure 4). These results may imply that **1** can inhibit the initial event of peripheral leukocyte influx into the cerebral parenchyma and activation of endogenous microglia after ischemia, which result in the reduction of cytokines, adhesion molecules, and iNOS. As a whole, these data suggest that the neurovascular protective effects of **1** after focal ischemia may be mediated in part through its anti-inflammatory properties.

AQP-4, localized in astrocyte foot processes near capillaries and in the ependymal cells lining the ventricles, is the major water channel in the CNS, which plays a key role in the generation of post-ischemic edema [18]. It was reported that AQP-4-deficient mice subjected to MCAO show better neurological outcomes than normal control mice, and the cerebral edema was 35% lower in AQP-4-deficient mice than in normal control mice [19]. We determined the expression of AQP-4 using Western blotting and immunohistochemistry methods at the two time points of 24 h and 7 d after MCAO surgery. Our results showed that **1** significantly inhibited the overexpression of AQP-4 at both time points. Whether this effect is a direct action or an indirect action following its anti-inflammation property needs further confirmation.

In addition to cytokines and cellular adhesion, leukocyte trafficking also affects the regulation of the tight junction complex, thus changing the BBB function [20,21]. Leukocyte recruitment following IL-1β injection into the brain can trigger signal transduction cascades leading to junctional disorganization with the breakdown of key components including occludin and ZO-1 [22]. In our experiment, there were significant decreases in mRNA expressions of occludin and ZO-1 at 24 h after MCAO in the model group when compared with the sham group. The decreases in mRNA expression of occludin and ZO-1 were inhibited by **1** dose-dependently. It suggests that **1** might protect the integrity of the BBB after ischemia partly by inhibiting the loss of tight junction proteins such as occludin and ZO-1.

MMPs have been shown to be involved in the degradation of the extracellular matrix, disrupting the BBB and allowing inflammatory cells to enter the brain [23–24]. Recently, it was demonstrated that MMP-9 knockout mice had significantly smaller lesion volumes compared with wild-type mice after permanent and transient focal ischemia, emphasizing the central role of this protease. A similar finding was obtained after transient global cerebral ischemia, with hippocampal neuron death being significantly ameliorated in MMP-9 knockout mice [16]. This study presents the first evidence that **1** treatment decreased MMP-9 protein levels in the brain after ischemia. One possible

mechanism of this inhibition might be due to the reduction of ROS levels by **1** treatment as reported previously, because ROS has been shown to be involved in MMP activation [24]. Whether the neurovascular protective effects of **1** are a direct consequence of MMP inhibition is being investigated in ongoing studies.

In conclusion, this study shows for the first time that **1** exerts its NVU protective action by inhibiting the inflammatory cascade following cerebral ischemia, preventing the integrity of the tight junction and BBB, downregulating the expressions of AQP-4 and MMP-9, and constructing the homeostatic balance. Because **1** has the potential to inhibit multiple mechanisms of injury after ischemia, it may be more effective than alternative candidate treatments that block only a single pathway. These findings of **1** are interesting and deserve further investigation for future clinical trials on stroke patients.

3. Experimental

3.1 Drugs and reagents

Racemic of 1 ((+)-pinocembrin: (−)-pinocembrin = 1:1, 99% purity) used in the present study was synthesized and processed as a sterile injection powder at the Department of New Drug Development, Institute of Materia Medica, Chinese Academy of Medical Sciences. It was dissolved in 0.9% NaCl before use. Antibodies of OX42, GFAP, MMP-9, iNOS, AQP-4, ICAM-1, VCAM-1, IL-1β, and TNF-α were obtained from Santa Cruz, CA, USA.

3.2 Experimental animals

Male Sprague–Dawley rats weighing 240–280 g obtained from Vital River Experimental Animal and Technology Co. Ltd (Beijing, China) were housed under a natural 12 h light–12 h dark cycle with food and water available *ad libitum.* All procedures were performed in accordance with institutional guidelines, and all efforts were made to minimize suffering and the number of rats used.

3.3 Experimental design and administration of drugs

After undergoing permanent MCAO, rats were randomly assigned one of four groups: model (vehicle-treated) group, 3, 10, and 30 mg/kg dose of **1** groups. Sham operation was performed for control rats by omitting only the occlusion. All drugs were administered via tail vein injection at 10 min, 8 h and 16 h after occlusion. The rats in the model and sham groups were administered the same volume of normal saline.

3.4 Focal cerebral ischemia model

Rats were anesthetized with chloral hydrate (Tianjin, China) intraperitoneally at a dose of 380 mg/kg. A rectal temperature probe was introduced, and a heating pad maintained the body temperature at 37 ± 0.5°C. Permanent focal brain ischemia model was induced by MCAO according to the described procedure [25]. Briefly, 4-0 monofilament nylon suture with a round tip was advanced from the right external carotid artery into the lumen of the internal carotid artery to occlude the origin of the MCA. Control rats were sham operated by omitting only the occlusion. The surgical procedure was completed in 15 min and did not involve significant blood loss.

3.5 Transmission electron microscopy studies

Animals were subjected to MCAO surgery. Twenty-four hours after the occlusion, rats were perfused with 0.9% saline and 4% paraformaldehyde for fixation. Then, the rats were decapitated and brains were immediately removed and cut into

adjacent 2 mm coronal sections. The specimens were multipoint sampled from cortical tissue in the MCA area, and then placed in 2.5% glutaraldehyde fixative for more than 2 h. The specimens were subsequently washed with 0.1 M phosphoric acid buffer three times. Osmium tetroxide (1%) was added afterwards to fix for 2 h at 4°C. Then, the specimens were dehydrated at room temperature in a series of 50, 70, 90, and 100% ethanol for 5 min each. Epoxypropane was used to permute at 1:1 and 1:4 ratios of epoxypropane and resin. Thereafter, the specimens were embedded in ethoxyline resin and polymerized: 35°C for 16 h, 45°C for 8 h, 55°C for 14 h and 60°C for 48 h. After trimming the dice, a half thickness slice was made. Each section was stained for 15 min in aqueous uranyl acetate, followed by 15 min in lead citrate. Finally, the sections were observed under an EM-1200EX transmission electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan). Assessments were made by an independent observer blinded to the study.

3.6 Western blot analysis

Fresh or frozen brain tissues were used for the Western blot analysis. Tissues (brain) were homogenized and lysed with ice-cold protein lysis buffer containing 10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μM aprotinin, 10 μM leupeptin, and 1 mM phenylmethyl sulfonyl fluoride for 30 min. The samples were centrifuged at 12,000*g* for 20 min to precipitate the protein. The dry pellets were then boiled for 5 min in loading buffer. Protein concentration was determined using the Bradford method. Prior to gel electrophoresis, samples were diluted with 1 mg/ml protein with equal volume of sample and lysis buffer, and 30 μl of each sample was loaded onto 4–20% gradient gels, which were electrophoresed at 200 V (Bio-Rad, Hercules, CA, USA). Proteins were then transferred to nitrocellulose membranes. Membranes were blocked in 3% milk powder in 0.1% Tween 20-phosphate-buffered saline for 1 h. Primary antibodies of iNOS, TNF-α, IL-1β, ICAM-1, VCAM-1, and AQP-4 (Santa Cruz, CA, USA) were diluted in blocking buffer and incubated with the membrane overnight at 4°C. Membranes were then incubated in a secondary antibody at a 1:5000 dilution for 1 h. Between steps, membranes were washed three times in Tris/Tween solution. Membranes were incubated with ECL Western blotting detection reagents (SuperECL Plus, Applygen Technologies Inc., Beijing, China) and exposed to an ECL imaging system (Fujifilm LAS-3000, Tokyo, Japan), and then densitometrically analyzed by an imaging system (Touching 2002 Gel Analysis software, Shanghai, China).

3.7 Immunohistochemistry

Expressions of microglia and astrocyte markers (OX42 and GFAP), MMP-9 at 24 h of MCAO, expressions of cytokines (TNF-α and IL-1β), iNOS, and AQP-4 7 days after MCAO were detected by immunohistochemical analysis using specific antibodies. Paraffin-embedded sections from the formalin-fixed brain tissues were stained for specific proteins. In brief, the brain tissue sections were deparaffinized, sequentially rehydrated in graded alcohol, and then immersed in PBS (pH 7.4). Slides were then microwaved for 2 min in antigen-unmasking solution (Vector Labs, Burlingame, CA, USA), cooled, and washed three times for 2 min in PBS. Sections were immersed for 25 min in 3% hydrogen peroxide in distilled water to eliminate endogenous peroxidase activity, and then blocked in immunohistochemical grade 1% bovine serum albumin in PBS for 1 h and diluted goat serum for 30 min to reduce non-specific staining. Sections were incubated overnight with primary antibodies. They were then rinsed three

times for 5 min in PBS containing 0.1% Tween-20. Secondary biotinylated anti-rabbit or anti-goat or anti-mouse IgG was incubated on slides for 30 min followed by an avidin–biotin HRP complex (Vectastain ABC-Elite Kit, Vector Labs) with DAB as the substrate. The slides were then dehydrated through a graded series of alcohol, mounted in Permount and coverslipped. All the sections were analyzed using an Olympus microscope and images were captured using a digital video camera controlled by Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

3.8 mRNA expression evaluated by RT-PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. RNA was quantified by optical density measurements at 260 and 280 nm. Integrity was confirmed by running samples on 1% agarose gel. One microgram of total RNA from each sample was used for cDNA synthesis. Reverse transcriptions were performed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the supplier's instructions. PCR was performed using TaqDNA polymerase obtained from Tiangen (Beijing, China). The following primers synthesized by Sangon (Sangon Biological Engineering Technology & Services Co. Ltd, Shanghai, China) were used for the PCR amplifications: occludin forward, 5′-GGGAATGTCCAGAACGAGAA-3′; occludin reverse, 5′-AACCGTACATAGATCCAGAAGC-3′; ZO-1 forward, 5′-TGAGCGGGCTACCTTATTGA-3′; ZO-1 reverse, 5′-CATCTGGCTGTCCGACTTGA-3′; GAPDH forward, 5′-GCCAAAAGGGTCATCATCTC-3′; GAPDH reverse, 5′-GGCCATCCACAGTCTTCT-3′. PCR was performed for 40 s at 94, 54, and 72°C, respectively. Products were inspected visually on 2% precast agarose gel with ethidium bromide staining (Invitrogen). Bands were quantified by densitometry. Counts for background were subtracted from counts for the specific bands for the occludin, ZO-1, or GAPDH signals. Ratios were calculated for occludin and ZO-1 signals with the control signals from GAPDH. Averages from these ratios were presented.

3.9 Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as means ± SEM. $P < 0.05$ was considered to be significant.

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