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Original article

Molecular mechanisms for N^G-nitro-L-arginine methyl ester action against cerebral ischemia–reperfusion injury-induced blood–brain barrier dysfunction

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Background: Ischemic stroke, an acute neurological injury lacking an effective therapy, is a leading cause of death worldwide. The unmet need in stroke research is to identify viable therapeutic targets and to understand their interplay during cerebral ischemia–reperfusion (I/R) injury.
Objective: To explore the protective effects and molecular mechanism of N^G-nitro-L-arginine methyl ester (L-NAME) in cerebral ischemia–reperfusion injury-induced blood–brain barrier (BBB) dysfunction.
Methods: Two hundred fifty-six rats were randomly assigned to a sham operation group, I/R group, and I/R with L-NAME treatment group. Brain water content was determined by calculating dry/wet weight. The permeability of the BBB was observed using an electron microscope and by determining the Evans Blue leakage from brain tissue on the ischemic side. The expression of brain MMP-9 and GFAP was determined using an immunohistochemical method. The expression of ZO-1 protein was determined by western blotting.
Results: We found that L-NAME remarkably attenuated the permeability of the BBB after I/R as assessed by Evans Blue leakage and brain water content ($p < 0.05$). This was further confirmed by examination of the ultrastructural morphology of the BBB using a transmission electron microscope. Furthermore, we found that expression of the zonae occludens-1 (ZO-1) was decreased in endothelial cells, and expression of MMP-9 and GFAP was increased in the basement membrane and astrocyte end-feet in vehicle control groups, respectively, but these changes could be prevented by L-NAME pretreatment.
Conclusion: These results suggested that the neuroprotective effects of L-NAME against BBB damage induced by I/R might be related to the upregulation of tight junction proteins and inhibition of MMP-9 and GFAP expression. L-NAME can be used as a potential MMP-9-based multiple targeting therapeutic strategy in cerebral I/R injury.

Keywords: blood–brain barrier, cerebral ischemia–reperfusion injury, glial fibrillary acidic protein, matrix metalloproteinase, N^G-nitro-L-arginine methyl ester, zonae occludens-1

Ischemic stroke, a leading cause of morbidity and mortality worldwide, is a major socioeconomic burden despite decades of concerted effort to find a suitable therapy [1-4]. Rapid reperfusion is the most effective treatment for ischemia, minimizing both structural and functional injuries. Paradoxically, however, restoration of cerebral blood flow causes further damage to the ischemic brain [5]. Therefore, protection against ischemia–reperfusion (I/R) injury remains a great challenge for stroke research. There is an urgent need to find new effective and safe treatments for ischemic stroke.

The blood–brain barrier (BBB) structure comprises three parts: tight junctions (TJ) between capillary endothelial cells; the basal lamina forming a distinct perivascular extracellular matrix and pericytes embedded within it; and the network surrounding the capillaries that are formed by astrocyte end-feet. Integrity of this barrier is ensured on the one hand by the basement membrane, which gives structural support to blood vessels, and by junction proteins in endothelial cells on the other.

Matrix metalloproteinases (MMPs, especially MMP-9), which are zinc-containing proteolytic

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enzymes that degrade components of the extracellular matrix and of basement membranes, have a central role in disease progression after I/R injury, as suggested by numerous studies using MMP inhibitors or MMP-deficient mice. TJs between endothelial cells form a metabolic and physical barrier restricting the movement of macromolecules between the blood and brain to maintain cerebral homeostasis. Tight junctions are vital to the structure and function of the BBB. The loss of zonae occludens-1 (ZO-1) could result in a disorganization of the TJs. ZO-1 is considered to contribute greatly to the post ischemic BBB breakdown after ischemic stroke [6, 7]. Activated MMPs can hydrolyze the BBB extracellular matrix and TJ proteins, degrade the extracellular matrix around cerebral blood vessels and neurons, and subsequently lead to BBB opening, brain edema, hemorrhage, and cell death [8, 9, 10]. Dejonckheere et al. [11] reviewed the use of MMP as drug targets, and proposed MMP-based multiple targeting therapeutic strategies in cerebral I/R injury.

Astrocytes, one class of glial cells, play a leading role in the modulation of synaptic transmission throughout the brain [12]. Glial fibrillary acidic protein (GFAP) is a major component of neurofilaments. Its overexpression is closely related to morphological alterations of astrocytes in response to neuronal damage. Astrocyte activation has been considered to be an important component of infarct progression [13]. However, few studies have examined the timing of MMP-9, GFAP, and tight junction-associated protein expression in light of permeability alterations associated with ischemia. Moreover, to our knowledge, a precise analysis of the expression of MMP-9, GFAP, and the tight junction-associated protein, ZO-1, has not been previously reported. The dynamic alterations of multiple BBB associated proteins during reperfusion injury remain not fully understood.

Administration of N^G-nitro-L-arginine methyl ester (L-NAME, a nonselective nitric oxide synthase (NOS) inhibitor) significantly reduced vascular damage, BBB permeability, and MMP-9 activity in cerebral I/R injury [14, 15, 16]. Stevanovic et al. suggested that NO action was included in the mechanism $AlCl_3$-induced neurotoxicity. L-NAME has a potential neuroprotective effect, which may inhibit immunoreactivity of astrocytes and the expression of GFAP in animals with aluminum toxicity [17].

Although L-NAME can exert multiple effects under pathophysiological conditions, the effects of L-NAME on cerebral I/R-induced disruption of the BBB have not yet been fully clarified. In view of the above, the present study was undertaken to examine whether there exists a potential protective effect of L-NAME on the structure and function of the BBB to support the maintenance of BBB integrity, and to explore the molecular mechanism of the L-NAME protective effect in a rodent model of cerebral I/R. Characterizing specific roles for these molecules in the BBB damage may be helpful to present molecular mechanisms and potential therapeutic strategies for ameliorating cerebral I/R damage.

Materials and methods

Animals and experimental groups

Adult male Sprague Dawley rats (220–260 g) were purchased from the Experimental Animal Center of Shanghai Jiaotong University School of Medicine. The animal procedures used this study were approved by the ethics review board of Shanghai First People's Hospital, School of Medicine, Shanghai Jiaotong University. Every effort was made to minimize the number of animals used and their suffering. The rats were housed in groups in laboratory cages and maintained at a controlled temperature (20 ± 2°C) under a 12 h light–dark cycle, with free access to food and water. Mean arterial blood pressure was monitored and core temperature was maintained at 37°C using a heating lamp during the surgery. Two hundred fifty-six rats were randomly divided into three groups: a sham operation group, I/R group, and I/R with L-NAME treatment group. Middle cerebral artery occlusion (MCAO) was maintained for 2 h, followed by 3, 6, 12, 24, and 48 h of reperfusion. Six rats were used for each time point and sham control for each group, except for the neurological experiments where 10 rats were used for each group and sham control for each group. In the L-NAME-pretreatment group, rats were injected intraperitoneally with 10 mg/kg L-NAME (Sigma, St Louis, MO, USA) at 15 min before nylon suture filament insertion for MCAO. Sham-operated and I/R rats were administrated an equivalent volume of saline.

Model of focal cerebral ischemia–reperfusion

Focal cerebral ischemia was induced by 2 h transient occlusion of the middle cerebral artery, as previously described in detail [18]. Briefly, rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.). After a median neck incision, the left common,

external, and internal carotid arteries were exposed. The common and external carotid arteries were permanently ligated with sutures. A nylon filament (diameter 0.26 mm) was then inserted into the common carotid artery and gently advanced into the internal carotid artery, approximately 18–20 mm from the carotid bifurcation, until the beginning of the middle cerebral artery in the circle of Willis. To allow reperfusion, the nylon filament was withdrawn 2 h after MCAO. In the sham-operated group, rats were exposed to the same surgical procedure, but without MCAO.

Brain water content

At 3, 6, 12, 24, and 48 h after MCAO, rats were killed and their brains were removed. The pons and olfactory bulb were removed and the brains were weighed to obtain their wet weight (ww). Subsequently the brains were dried at 110°C for 24 h to determine their dry weight (dw). Brain water content was calculated by using the following formula: ((ww – dw)/ww) × 100 and the result was used as an index for brain edema [19].

Measurement of blood–brain barrier permeability

The permeability of the BBB was quantitatively determined by extravasation of Evans Blue dye as a marker of albumin extravasation [20, 21]. Briefly, 2% Evans Blue in saline (2 mg/kg) was injected intravenously 2 h before each expected time point via a femoral vein. At the expected time after operation, rats were deeply anesthetized with 10% chloral hydrate and infused with heparinized saline through the cardiac ventricle until colorless infusion fluid was obtained from the atrium. After the rats had been sacrificed by decapitation, the brain hemispheres were separated along the sagittal suture. Then, each hemisphere was weighed and put into formamide (1 ml/100 mg) at 60°C for 24 h. The concentration of dye extracted from each brain hemisphere was determined using spectrophotometry at 620 nm. The quantitative calculation of the dye content in the brain was based on the external standards dissolved in the same solvent. Meanwhile, gradient concentrations of Evans Blue were used for a standard curve. BBB leakage was represented as μg per gram brain parenchyma.

Transmission electron microscopy

The ultrastructure of BBB was examined using transmission electron microscopy (TEM). Rats were deeply anesthetized with 10% chloral hydrate. The heart was exposed and the left ventricles were perfused with 0.9% saline via a catheter through the aortic artery until colorless infusion, followed by perfusion with a fixative consisting of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Brains were resected and the frontal and parietal cortex of ischemic brain tissues were chosen as samples. The samples were divided into pieces of 1 mm^3, and fixed with 2.5% glutaraldehyde at 4°C. Following standard procedures, semi- and ultrathin sections were prepared, and stained with uranyl acetate and lead citrate. Then BBB changes were examined by TEM (JEM-1200EX; JEOL, Tokyo, Japan).

Immunohistochemistry assay

Immunohistochemistry was used to detect the distribution and expression of MMP-9 and GFAP in brain tissues of cerebral I/R and sham-operated rats. Rats were deeply anesthetized and perfused with heparinized saline and 4% paraformaldehyde. Their brains were postfixed for 24 h, and then immersed in 30% sucrose solution with phosphate-buffered saline for 24 h. Coronal sections at the level of the anterior commissure in the region of the infarct tissue were cut into pieces 10 μm thick on a cryostat at –25°C. The sections were stained with hematoxylin and eosin to visualize neuronal damage after I/R. Serial sections were used alternatively for MMP-9 or GFAP immunohistochemistry. They were rehydrated in PBS, then endogenous peroxidase activity was inactivated by incubation with 3% H_2O_2 solution for 10 min at room temperature. After blocking nonspecific binding sites on the sections with normal goat serum for 30 min, the sections were incubated with rabbit polyclonal anti-MMP-9 antibody (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-GFAP antibody (1:150; Santa Cruz Biotechnology) at 4°C overnight, respectively. Negative controls included omission of the primary antibody and replacing it with PBS during the procedures. An anti-rabbit IgG–peroxidase conjugate was used as a secondary antibody and localized using diaminobenzidine as a chromogen following standard procedures. For semi-quantitative measurements of MMP-9 and GFAP expression, the slides were photographed and OD at $\lambda_{570\ nm}$ analyzed using a computer-aided image-analyzing system (Motic Images Advanced software, version 3.2; Xiamen, China).

Western blot analysis of ZO-1

Thirty rats per group (I/R and L-NAME + I/R) were sacrificed at 3, 6, 12, 24, and 48 h after reperfusion, 6 sham controls at t = 0 were used per group. Their brains were carefully removed, placed in chilled saline, dissected into the penumbra and then snap-frozen in liquid nitrogen. For sample preparation, the tissue was homogenized in buffer with a protease inhibitor (Sigma). The samples were separated by electrophoresis on 12% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). After transfer to a PVDF membrane, nonspecific binding sites on membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.5% Tween 20 overnight at 4°C followed by incubation with polyclonal rabbit anti-ZO-1 (diluted 1:500, Zymed, South San Francisco, CA, USA). β-Actin was used as a loading control. After the membrane blots were the incubated with anti-rabbit secondary antibody for 1 h at room temperature, the protein signals were visualized using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Neurological deficit

Neurological de□cit was determined in an independent manner with observers blinded to treatment, as previously described [18]. Scoring was assigned as follows: normal motor function = 0, failure to extend right paw fully = 1, contralateral circling when held by the tail on a □at surface, though normal at rest circling to the right = 2, contralateral leaning when at rest = 3, no spontaneous motor activity = 4.

Statistical analyses

The results were collected from independent experiments. Data are presented as means ± standard error of the mean (SEM). Differences between groups were assessed using a one-way analysis of variance (ANOVA) followed by a least signi□cant difference test. Statistical analyses were performed using statistical software (SPSS version 18.0; Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Effect of L-NAME on blood–brain barrier integrity after ischemia–reperfusion

To measure the effect of L-NAME on BBB integrity after focal cerebral ischemia, we determined the Evans Blue dye content of brain parenchyma. As shown in **Figure 1**, the Evans Blue dye content was markedly increased in I/R group rat brains by comparison with the sham operated group rats ($p < 0.01$ at 24 and 48 h). In animals pretreated with L-NAME, the increase in Evans Blue content induced by I/R was signi□cantly attenuated.

Effect of L-NAME on brain water content after I/R

A total of 72 rats (n = 6 per data point) were used for the analysis of brain water content. Compared with the sham group, brain water content after MCAO was signi□cantly increased in I/R rats. Compared with the I/R group rats, brain water content after MCAO was signi□cantly less in rats that received L-NAME pretreatment (**Figure 2**).

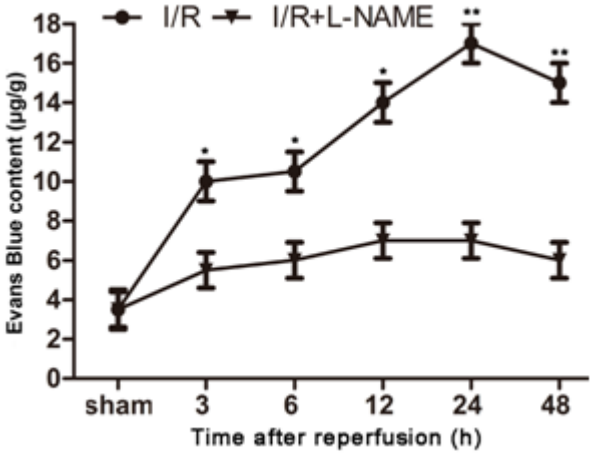

Figure 1. Effects of L-NAME pretreatment on Evans Blue content of the rat brain parenchyma (μg per g) after ischemia–reperfusion (I/R). Data represent the mean ± SEM. (n = 6 each data point). The Evans Blue content of the brain parenchyma was significantly increased in the I/R group by 3 h ($p < 0.05$) and greatly increased by 24 h after I/R ($p < 0.01$) compared with the L-NAME-treatment group. The Evans Blue content of the brain parenchyma was markedly less in the L-NAME-treatment group. *$p < 0.05$,**$p < 0.01$.

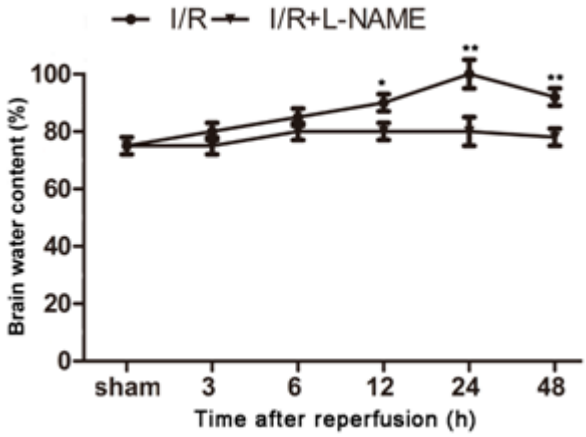

Figure 2. Effect of L-NAME pretreatment on water content of the brain parenchyma after ischemia–reperfusion (I/R) in rats. Data represent the mean ± SEM. (n = 6 each data point). The brain water content was significantly increased in the I/R group by 3 h ($p < 0.05$) and greatly increased at 24 h after I/R ($p < 0.01$). The brain water content was markedly less in the L-NAME treatment group. $*p < 0.05$,$**p < 0.01$.

Transmission electron microscopic examination of effect of L-NAME on blood–brain barrier integrity

EM showed that cerebral microvessels in sham-operated rats exhibited capillary integrity with normal endothelial cells, basal laminae, and astrocyte end-feet. In the I/R group, the endothelial cells and their nuclei were swollen and deformed, and the lumen of capillary was collapsed (**Figure 3A1, A2**). The number of synapses and synaptic vesicles decreases, mitochondria of astrocytes of the cerebral cortex were swollen and vacuolated. The integrity of the BBB was destroyed, presenting perivascular edema, vacuolation, and membrane damage (**Figure 3B1 and B2**). In the L-NAME-pretreatment group, nuclear chromatin was condensed slightly, and the structures of the endothelial cells and neurosynapses were preserved (**Figure 3C1, C2**).

Immunohistochemical analysis of MMP-9 and GFAP

The number of MMP-9- and GFAP-immuno-reactive cells was increased in the I/R group, and was signi□cantly different compared with those in the sham groups (**Figures 4–6** and **Table 1**). L-NAME pretreatment markedly downregulated the expression of MMP-9 and GFAP by contrast with the I/R group, although the downregulation of the proteins was not reduced to sham-operated control levels (**Figure 4B1 and B2** and **Figure 5B1, B2, and B3**).

L-NAME changed the distribution and protein expression of ZO-1 following MCAO

Western blotting (**Figure 7**) showed ZO-1 expression levels were significantly elevated in the L-NAME-pretreated groups compared with the saline-pretreated I/R groups at 3, 6, 12, 24, and 48 h ($p < 0.05$). The expression of ZO-1 was considerably less in the I/R group rats (**Figures 7 and 8**).

Neurological function

Neurological function was signi□cantly decreased in the I/R group after MCAO compared with the sham group. Pretreatment with L-NAME remarkably lessened functional defects from 6 h up to at least 48 h after MCAO (**Figure 9**).

Discussion

Cerebral ischemia provokes an irreversible neurodegenerative disorder that may lead clinically to progressive dementia and global cognitive deterioration. Increased vascular permeability and disruption of the BBB may be initiating factors for the development of cerebral infarctions [22, 23]. Complicated pathophysiological progresses and multiple mechanisms are involved in I/R injury, including cerebral edema, and hemorrhagic transformation [24, 25]. Therefore, the development of agents that provide effective neuroprotection against reperfusion injury is warranted.

The BBB is a protective membranous barrier that restricts the entry of molecules and white blood cells from the systemic circulation into the central nervous system (CNS). It functions to maintain homeostatic balance of the extracellular fluid in the brain, thereby ensuring normal brain function. It is well known that the BBB is composed of a continuous layer

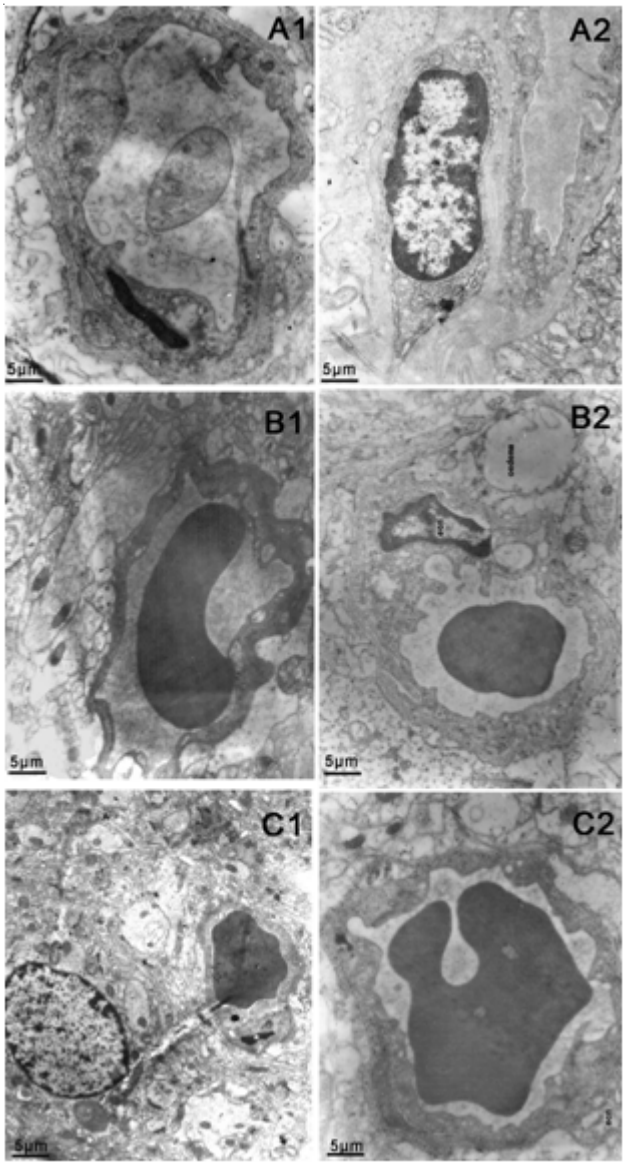

Figure 3. Effects of L-NAME pretreatment on the ultrastructural morphology of the blood–brain barrier after ischemia–reperfusion. **A1 and A2:** Under sham conditions, endothelial cells, basal laminae, and astrocyte end-feet were intact; **B1 and B2:** At 48 h after reperfusion, the endothelial cells were swollen and deformed, the lumen of capillary was collapsed, and the number of synapses was decreased; **C1 and C2:** After L-NAME treatment, the structure of BBB was preserved. Scale bars = 5 μm.

of brain microvascular endothelial cells together with pericytes, a basal lamina, and astrocytic end-feet. Activated MMPs are responsible for degradation of the extracellular matrix around cerebral blood vessels and neurons and increase the permeability of the BBB by hydrolyzing the BBB extracellular matrix and tight junction proteins. Tight junctions between endothelial cells form a metabolic and physical barrier restricting the movement of macromolecules between the blood and brain to maintain cerebral homeostasis [6]. The astrocytic end-feet function in brain water homeostasis, which together with the pericytes, have been implicated in BBB development and permeability, although their precise role in the BBB remains disputed [26-28]. Therefore, protection of the BBB in brain tissues may be beneficial for neuronal recovery from ischemic/reperfusion injury.

We found that pretreatment of rats with L-NAME before MCAO maintained the integrity of BBB as shown by a lower Evans Blue dye and water content

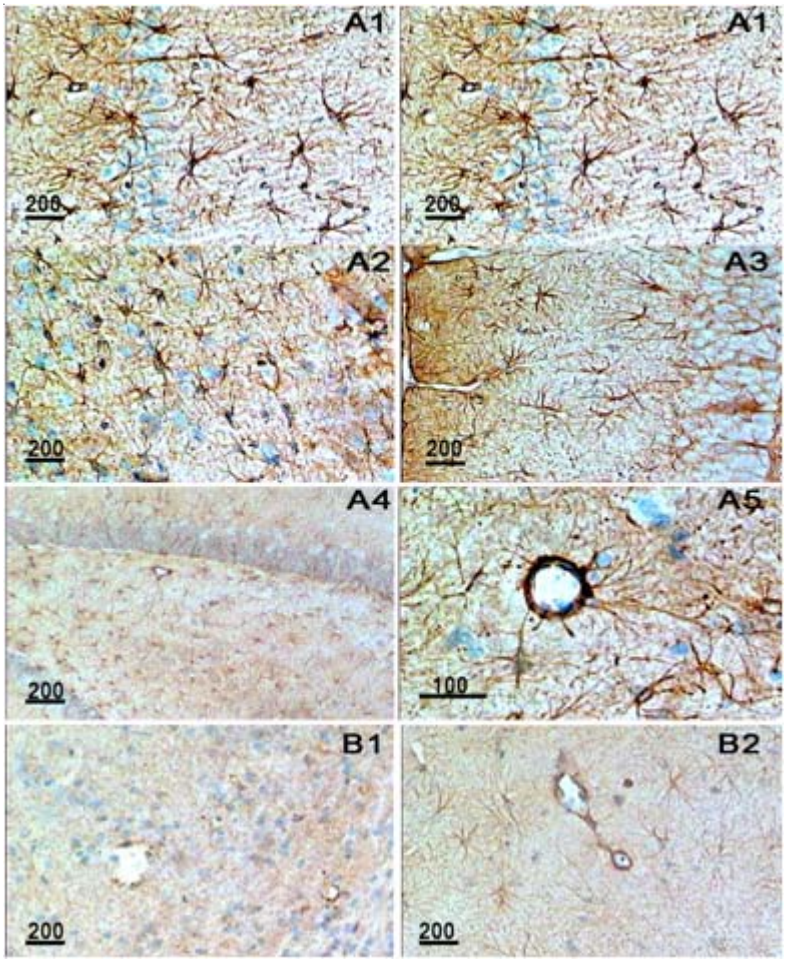

Figure 4. Representative GFAP immunohistochemistry of the ipsilateral hemisphere after cerebral ischemia–reperfusion (I/R) injury. Sham-operated rats showed very low levels of activated GFAP, while the levels were increased in the I/R group at 6, 12, 24, and 48 h (A1 [3 h], A2 [6 h], A3 [12 h], A4 [24 h], A5 [48 h]. L-NAME pretreatment prevented the I/R-induced increase in expression in the ipsilateral hemisphere (B1 [24 h], B2 [48 h]). A5 ×400; A1, A2, A3, A4, B1, B2 ×200.

in the brain parenchyma after cerebral I/R. EM showed that L-NAME markedly prevented endothelial cell damage, attenuating the swelling of the basement membrane, maintained synaptic connections, and avoided astrocyte vacuolation. Furthermore, an obvious avoidance of neurological deterioration was observed in L-NAME-pretreated rats was seen 3, 6, 12, 24, and 48 h after I/R. To our knowledge, the present study provides the first evidence of L-NAME in protection of the BBB against I/R-induced increase in the BBB permeability and subsequent neurological deficit.

Reactive astrocyte activation, including an increase of size and number, is involved in the histopathology of ischemia. Activation of reactive astrocytes increases Src immunoreactivity and consequently exacerbates ischemic injury [29]. It is firmly established that astrocytes also regulate synaptic function throughout the brain [30]. As the predominant intermediate filament protein in astrocytes of mammalian CNS, the astrocyte differentiation marker GFAP has been widely used to evaluate the 'reactive state' of astrocytes. A dramatic elevation of GFAP was reported in model of focal cerebral ischemia when ischemic injury was confined to the cerebral cortex [31]. This indicates that pharmacological intervention affecting astrocytes may ameliorate the ischemic insult. The current study demonstrated that the immunoreactivity of GFAP was significantly less after pretreatment of rats with L-NAME, implying that L-NAME is neuroprotective against cerebral I/R injury and could be correlated with inhibiting astrogliosis. However, the underlying mechanism by which L-NAME suppresses astrocytic activation remains to be explored further.

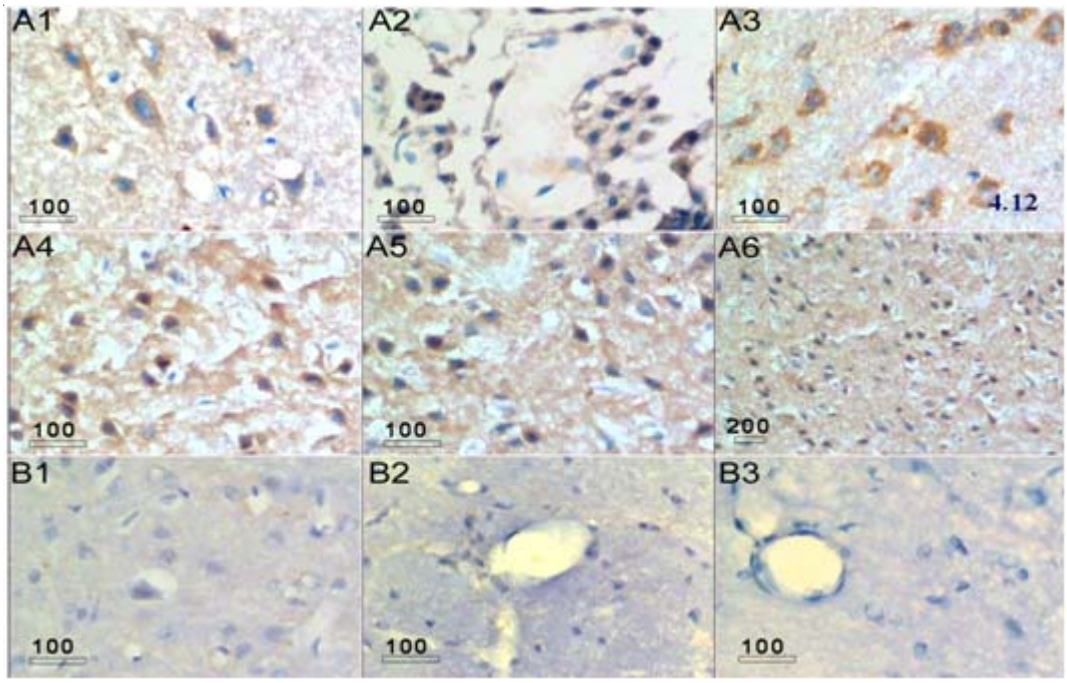

Figure 5. Representative MMP-9 immunohistochemistry of the ipsilateral hemisphere after cerebral ischemia–reperfusion (I/R) injury. Sham-operated rats showed very low levels of activated MMP-9, while the levels were increased in the I/R group at 6, 12, 24, and 48 h (A1, A2 [6h], A3 [12 h], A4, A5 [24 h], A6 [48 h ×200]). L-NAME pretreatment prevented the I/R-induced increase in expression in the ipsilateral hemisphere (B1 [12 h], B2 [24 h], B3 [48 h]). A6 ×200; A1, A2, A3, A4, A5, B1, B2, B3 ×400.

Table 1. Comparison of MMP-9- and GFAP-positive cell numbers between groups

Group	MMP-9 positive cells/cm^2	GFAP positive cells/cm^2
sham	4.2 ± 0.2	5.0 ± 1.9
I/R		
3 h	15.6 ± 1.7*	29.4 ± 3.7*
6 h	37.6 ± 1.6*	30.7 ± 3.8*
12 h	40.2 ± 1.3*	39.2 ± 3.6*
24 h	46.0 ± 1.2*	36.9 ± 3.4*
48 h	38.9 ± 1.3*	37.1 ± 3.2*
I/R + L-NAME		
3 h	10.5 ± 1.6$^{\#}$	13.5 ± 3.6$^{\#}$
6 h	23.6 ± 1.3$^{\#}$	16.4 ± 3.3$^{\#}$
12 h	27.5 ± 1.4$^{\#}$	17.3 ± 3.1$^{\#}$
24 h	24.6 ± 1.5$^{\#}$	15.1 ± 3.2$^{\#}$
48 h	21.5 ± 1.4$^{\#}$	12.6 ± 3.2$^{\#}$

(mean ± SEM, n = 6)
*$p < 0.01$ vs. sham group, $^{\#}p < 0.05$ vs. I/R group

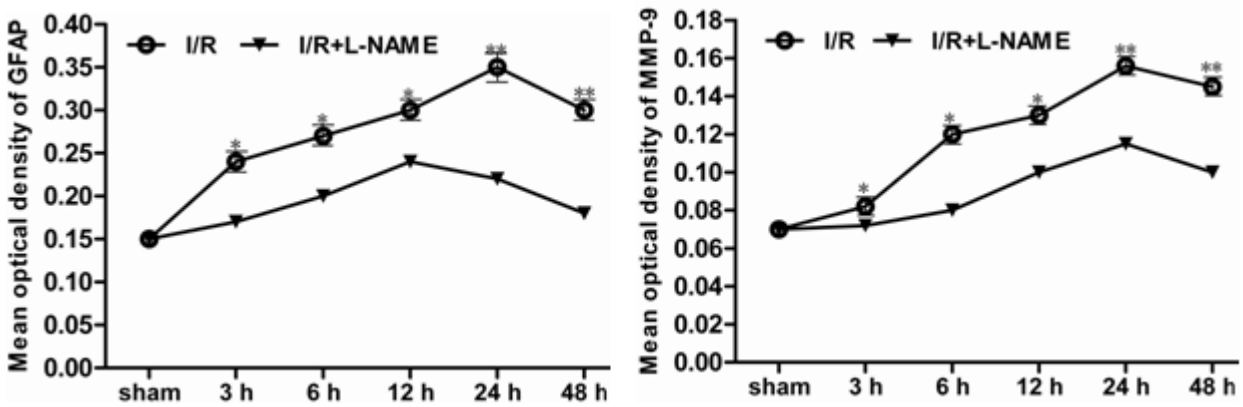

Figure 6. Mean optical density (OD) of MMP-9 and GFAP staining (OD/mm^2 at $\lambda_{570\ nm}$). The I/R-induced increase in the OD of the MMP-9 and GFAP was attenuated by L-NAME administration (n = 6). *$p < 0.05$,**$p < 0.01$.

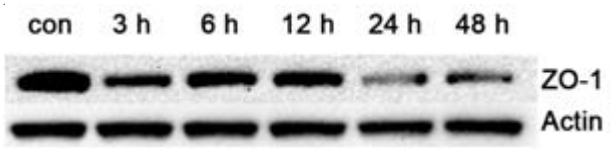

Figure 7. Western blotting showing changes of ZO-1 levels in the brain parenchyma of L-NAME pretreated rats after I/R injury.

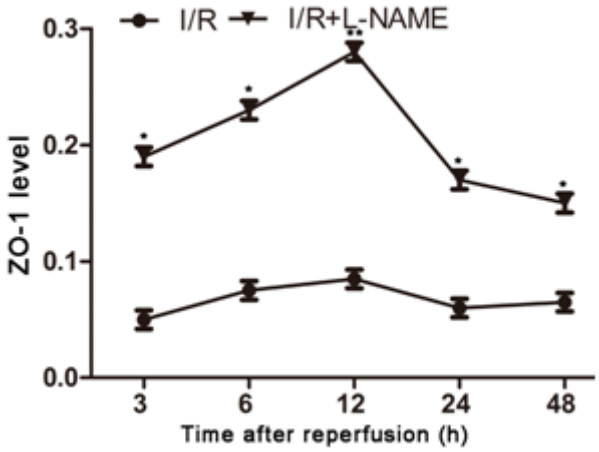

Figure 8. Western blotting ECL densitometry results showing changes of ZO-1 levels in rat brain parenchyma relative to β-actin loading controls after I/R. *$p < 0.05$,**$p < 0.01$.

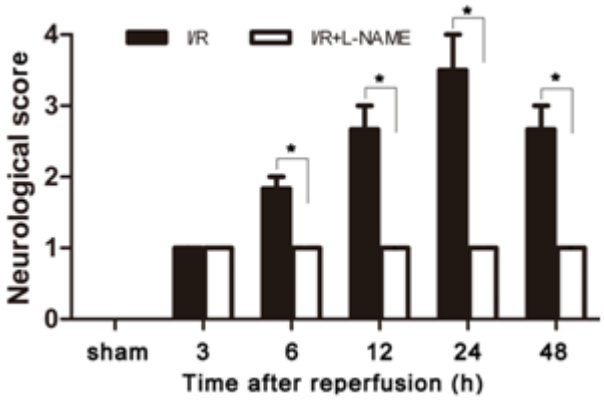

Figure 9. Neurological function. Scoring was assigned as follows: 0 = normal motor function, 1 = failure to extend right paw fully, 2 = contralateral circling when held by tail on □at surface, though normal at rest circling to right, 3 = contralateral leaning when at rest, 4 = no spontaneous motor activity. Neurological scores were significantly higher after MCAO and L-NAME pretreatment had a significant neuroprotective effect compared with the I/R group rats from 6 h up to at least 48 h after MCAO ($p < 0.01$, n = 10 per group).

Disruption of the BBB is a critical event during cerebral ischemia, followed by passive diffusion of water leading to vasogenic edema and secondary brain injury, in which MMP-9 plays a pivotal role. MMP-9 is produced in endothelial cells, microglia and astrocytes, and is upregulated after cerebral I/R injury in experimental animals [32] and in human patients [33]. Suofu et al. [34] suggested that target MMPs might help us to protect the postischemic brain from injury and hemorrhagic transformation. Consistent with this suggestion, our study showed that MMP-9 was rapidly upregulated in rats after cerebral I/R injury, and corresponded to sequential disruption of the BBB. This finding supports the involvement of MMP-9 in the BBB breakdown after cerebral I/R injury [35-37]. Further, L-NAME pretreatment ameliorated the disruption of the BBB after ischemic stroke and this correlated with reduced MMP-9 protein levels and enzyme activity, suggesting that L-NAME could protect the BBB during cerebral ischemia reperfusion injury through an MMP9-dependent mechanism.

Moreover, the current study provides insight into the mechanism of BBB function preservation in cerebral ischemia by L-NAME pretreatment. Tight junctions are vital to the structure and function of BBB. Disruption of the tight junction barrier may be directly involved in the pathogenesis and aggravation of cerebral I/R injury. ZO-1 is a major constituent of tight junction barrier formation [26]. In our present study, the higher expression of ZO-1 in the L-NAME-pretreated group than in the vehicle group implies that ZO-1 is involved in the mechanism whereby L-NAME protects the BBB against I/R-induced dysfunction. The mechanism apparently involves upregulating the expression of ZO-1 and downregulating expression of MMP-9. Our current results are consistent with a previous study describing the reestablishment of the TJ barrier and inhibition of MMP-9 activation in protection against cerebral I/R injury in rats [38].

Our study is limited in that we had not used a sham control for every time point. The changes in sham controls at time points after reperfusion needs to be explored further.

Conclusion

The present study demonstrated that inhibition of NOS by L-NAME, which in turn decreases NO production, could diminish neurological dysfunction and offer signi□cant protection against the breakdown of the BBB after cerebral I/R. These actions may be mediated by downregulating MMP-9 expression at the basal lamina and GFAP expression on astrocytic end-feet, and upregulating the expression of the tight junction protein ZO-1 in endothelial cells. L-NAME may potentially be used as an agent to protect against cerebral I/R injury. More detailed examination of the mechanisms of L-NAME-mediated neuroprotection is warranted.

Acknowledgments

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