

Time-course investigation of blood–brain barrier permeability and tight junction protein changes in a rat model of permanent focal ischemia

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Abstract Permanent middle cerebral artery occlusion (pMCAO) is an animal model that is widely used to simulate human ischemic stroke. However, the timing of the changes in the expression of tight junction (TJ) proteins and synaptic proteins associated with pMCAO remain incompletely understood. Therefore, to further explore the characteristics and mechanisms of blood–brain barrier (BBB) damage during cerebral ischemic stroke, we used a pMCAO rat model to define dynamic changes in BBB permeability within 120 h after

ischemia in order to examine the expression levels of the TJ proteins claudin-5 and occludin and the synaptic proteins synaptophysin (SYP) and postsynaptic density protein 95 (PSD95). In our study, Evans blue content began to increase at 4 h and was highest at 8 and 120 h after ischemia. TTC staining showed that cerebral infarction was observed at 4 h and that the percentage of infarct volume increased with time after ischemia. The expression levels of claudin-5 and occludin began to decline at 1 h and were lowest at 8 and 120 h after ischemia. The expression levels of SYP and PSD95 decreased from 12 to 120 h after ischemia. GFAP, an astrocyte marker, gradually increased in the cortex penumbra over time post-ischemia. Our study helps clarify the characteristics of pMCAO models and provides evidence supporting the translational potential of animal stroke models.

Keywords Cerebral ischemia · Blood–brain barrier · Tight junctions · pMCAO

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Introduction

Cerebral ischemia is a major cause of death and is associated with a high incidence of acquired disability and morbidity in adults [1, 2]. Intracranial thrombus-induced occlusion of the major cerebral artery, which decreases or completely blocks the blood flow in a specific brain region, is the pathogenesis of cerebral ischemia. Then, neurochemical and molecular events, including glutamate excitatory toxicity, Ca²⁺ overload, oxidative stress damage, the release of inflammatory cytokines, and neuronal death, lead to blood–brain barrier (BBB) leakage and brain tissue damage [3–7].

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Structural and functional changes in brain microvascular endothelial cells and tight junctions (TJs) between the endothelial cells regulate BBB permeability. TJs are composed of transmembrane proteins, among which claudin-5 and occludin are the most important for BBB integrity. Decreased expression of TJ mRNAs and proteins directly contributes to BBB breakdown, increased vascular permeability, vasogenic edema resulting in high intracranial pressure, and high mortality risk after ischemic stroke [8–10]. Up-regulation of TJ proteins is a potential therapeutic strategy for brain stroke.

Transient and permanent middle cerebral artery occlusion (tMCAO and pMCAO) models are the animal models most commonly used to mimic human ischemic stroke. Therefore, it is important to understand the different pathophysiological changes and characteristics of these two models for evaluating novel therapeutic agents to treat human ischemic stroke. In the tMCAO model, there may be recovery from the primary core damage, and secondary delayed cell death develops after an interval of 6–12 h. However, the therapeutic time frame for human stroke is shorter than this. By contrast, in the pMCAO model, primary core damage peaks at approximately 3 h after MCAO [11, 12]. Jiao et al. previously investigated changes in BBB integrity, endothelial morphology, and TJ proteins over time following tMCAO in SD rats [9]. However, the timing of the changes in TJ proteins and synaptic proteins associated with pMCAO remain unclear. In this study, we use the permanent model of ischemic stroke, which eliminates the influence of recanalization to investigate changes in BBB integrity, neuronal damage, astrocyte activity, and TJ proteins over time following ischemic stroke.

Materials and methods

Animal model subjects

Adult male SD rats (200–250 g) were procured from Liao Ning Chang Sheng Biotechnology Co., Ltd. The animals were maintained under standard housing conditions (room temperature 25 ± 1 °C and humidity $50 \pm 2\%$) with a 12-h light and 12-h dark cycle. Water and food were available ad libitum. All animal studies were performed in strict accordance with legislation in P.R. China on the use and care of laboratory animals and with guidelines established by the Institute for Experimental Animals at Shenyang Pharmaceutical University (Permit Number: SYPU-IACUC-C2015-0831-203). The rats were randomly divided into eight groups: sham-operated group and groups receiving pMCAO for 1, 4, 8, 12, 24, 72, or 120 h.

pMCAO

The rats were anesthetized with chloral hydrate (300 mg/kg i.p.). Then, the right common (CCA), external (ECA), and internal carotid artery (ICA) were exposed and isolated. A monofilament nylon suture with a blunted tip (0.26–0.28 mm) was inserted into the right ECA and pushed into the ICA until light resistance was felt. The filament was inserted approximately 19–22 mm in order to block the middle cerebral artery (MCA). The filament remained inserted for 1, 4, 8, 12, 24, 72, or 120 h, after which the animals were sacrificed. Sham group rats received the same surgery without insertion of the monofilament nylon suture.

Assessment of infarct size

Brain infarct size was evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) at 1, 4, 8, 12, 24, 72, and 120 h after pMCAO. The rats were sacrificed, and the brains were quickly isolated. Brains were sectioned into five coronal slices (3-mm intervals), stained with a 2% solution of TTC at 37 °C for 15 min, and then fixed with 4% paraformaldehyde. The infarct area appeared white, and the normal area was stained red. TTC-stained sections were photographed using a digital camera, and images were analyzed using Image-Pro Plus 5.1 software. The infarct area is expressed as the ratio of the volume of the white area over that of the total brain area. The total infarct size is expressed as the percentage of damaged area of five slices over the total brain area.

Measurement of BBB permeability

One hour before decapitation of the rats, 2% Evans blue (EB, 4 mg/kg) was injected into the tail vein. The rats were anesthetized with chloral hydrate (300 mg/kg i.p.), and then normal saline was injected into the left cardiac ventricle until colorless perfusion fluid was obtained from the right atrium. The right hemisphere of the brain was exposed and isolated. Then, each hemisphere was weighed, homogenized in 2 ml 5% trichloroacetic acid, and centrifuged (10,000 r/m, 20 min). The content of EB was determined via spectrophotometry (at 620 nm). The results are expressed as ng/g tissue, as calculated using a standard curve.

Western-blot analysis

The rats were decapitated under excessive ether anesthesia. Ischemic penumbra tissues were isolated and stored at -80 °C. Brain tissues were homogenized in 400 μ l of cold extraction buffer. Protein concentration of the lysate was

determined using a BCA Protein Assay Kit (Kangwei Biotechnology, China). Equal amounts of proteins (45 μ g) were separated by 8–10% sodium dodecyl sulfate polyacrylamide gels, electrophoretically transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated overnight at 4 °C with anti-Occludin antibody (1:500, Santa Cruz, USA), anti-Claudin-5 antibody (1:500, Santa Cruz, USA), anti-SYP antibody (1:1000, Santa Cruz, USA), anti-PSD95 antibody (1:1000, Santa Cruz, USA), anti-GFAP antibody (1:500, Boster, China), or anti- β -actin antibody (1:1000, Santa Cruz, USA). After the protein was stained for 2 h at room temperature with HRP-conjugated secondary antibodies against mouse IgG and rabbit IgG (1:5000, Santa Cruz, USA), an ECL kit (Kangwei Biotechnology, China) was used to detect protein bands. The intensities of the bands were quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad, USA) and corrected using the corresponding β -actin levels. The results are expressed as percentages of the control.

Statistical analysis

All data are presented as the mean \pm SEM. Statistical significance was determined with one-way ANOVA followed by Fisher’s least significant difference (LSD) multiple comparisons test; $p < 0.05$ was considered to indicate statistical significance. The data were analyzed using SPSS 17.0.

Results

Changes in infarct volume after ischemia

Brain infarct size was evaluated by TTC staining at 1, 4, 8, 12, 24, 72, and 120 h after ischemia. The infarct area appeared white, whereas the normal brain area was stained in red. Compared with the sham group, the infarct size percentage was significantly increased by 22.48 ± 3.52 , 25.60 ± 2.57 , 29.90 ± 1.33 , 35.11 ± 2.66 , and $28.50 \pm 1.86\%$ in the 8, 12, 24, 72, and 120 h groups, respectively ($p < 0.05$, Fig. 1). No significant infarct area was observed in the Sham group or in the 1 h post-pMCAO group. These results indicated a time-dependent increase in brain infarct injury after focal ischemia and that the infarct regions developed in both the striatum and lateral cortex.

Changes in BBB permeability after ischemia

The amount of EB leakage was measured in order to evaluate BBB permeability after ischemia in the pMCAO model. Increased EB content was observed in the ischemic regions, with the content significantly greater in the 4, 8, 12, 24, and

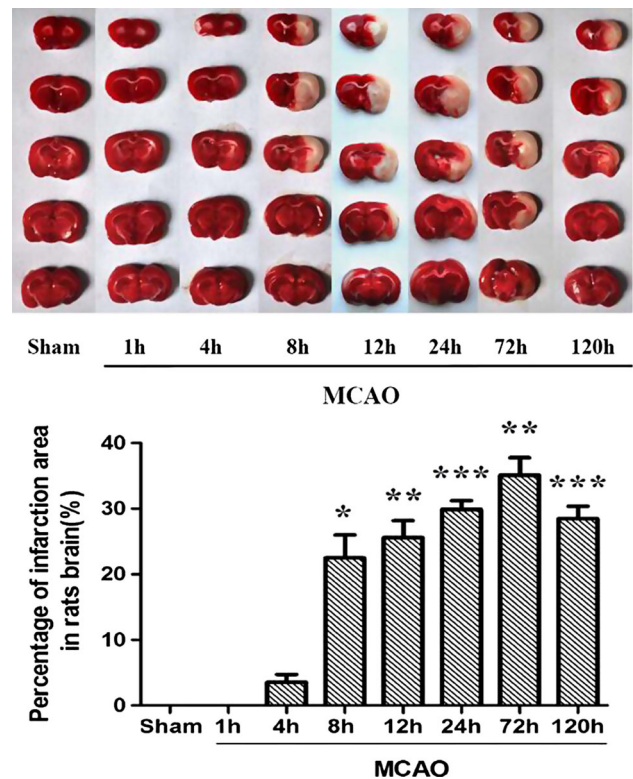


Fig. 1 Percentage of infarction area in rats brain at different time points after MCAO. Compared with the sham group, the infarct size percentage of the 8, 12, 24, 72, and 120 h groups were significantly increased. Data represent mean \pm SEM, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham group

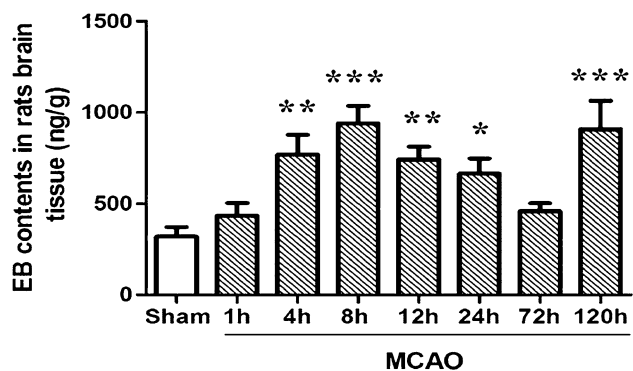


Fig. 2 Evans blue (EB) contents in brain tissue at different time points after MCAO. EB content of the 4, 8, 12, 24, and 120 h groups was significantly increased compared to that of the sham group. Data represent mean \pm SEM, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham group

120 h groups than in the sham group ($p < 0.05$, Fig. 2). EB content was highest at 8 and 120 h after ischemia.

Protein expression of SYP and PSD95 after ischemia

To assess pMCAO-induced neuronal damage, we measured the expression levels of the synaptic proteins SYP and

PSD95 in ischemic penumbra tissues. Expression levels of SYP at 8, 12, 24, 72, and 120 h after ischemia and of PSD95 at 12, 24, 72, and 120 h after were significantly lower than in the sham group ($p < 0.01$, Fig. 3a, b).

Protein expression of claudin-5 and occludin after ischemia

To assess pMCAO-induced BBB breakdown, we measured the expression levels of the TJ proteins claudin-5 and occludin in ischemic penumbra tissues. Expression levels

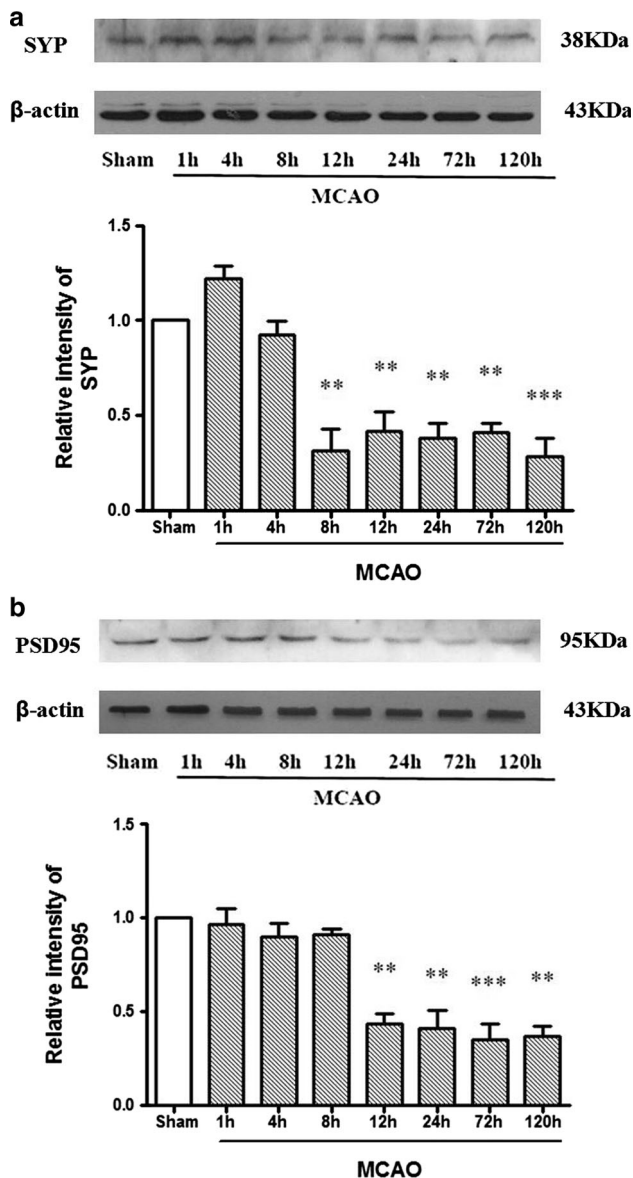


Fig. 3 The expression of SYP and PSD95 in penumbra at different time points after MCAO. Expression levels of SYP at 8, 12, 24, 72, and 120 h after ischemia and of PSD95 at 12, 24, 72, and 120 h after were significantly reduced compared to the sham group. Data represent mean \pm SEM, $n = 3$. ** $p < 0.01$, *** $p < 0.001$ vs. sham group

of claudin-5 at 8, 12, and 120 h after ischemia and of occludin at 8, 12, 24, and 120 h after were significantly lower than in the sham group ($p < 0.05$, Fig. 4a, b). These data show that the integrity of the BBB was lost in ischemic rats.

Protein expression of GFAP after ischemia

To examine the activity of astrocytes, we used Western blotting to measure the expression of GFAP. Expression levels of GFAP at 12, 24, and 120 h after ischemia were significantly greater than in the sham group ($p < 0.05$, Fig. 5).

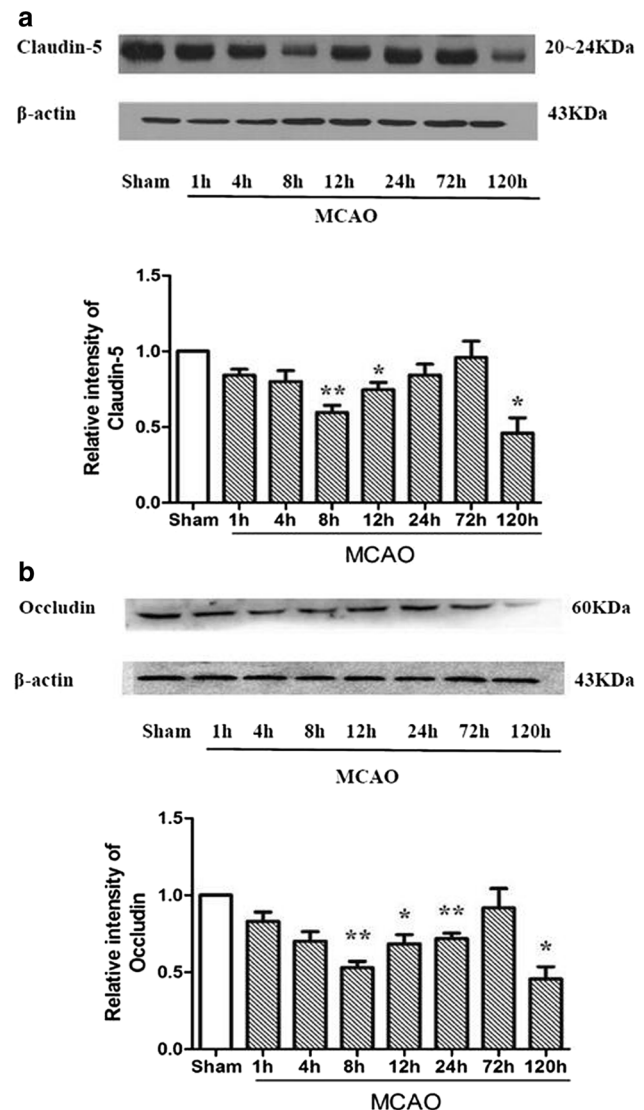


Fig. 4 The expression of Claudin-5 and Occludin in penumbra at different time points after MCAO. Expression levels of claudin-5 at 8, 12 and 120 h after ischemia and of occludin at 8, 12, 24, and 120 h after were significantly reduced compared to the sham group. Data represent mean \pm SEM, $n = 6$. * $p < 0.05$, ** $p < 0.01$ vs. sham group

Discussion

In this study, we demonstrated that BBB permeability in the ischemic hemisphere of the brain gradually increased within 120-h post-ischemia. Previous studies have reported that astrocytes play key roles in maintaining the integrity and promoting the maturation of BBB [13, 14]. TJs between endothelial cells play a key role in determining vascular permeability [15, 16]. Therefore, we characterized the time-course and relationship of the expression of the TJ proteins claudin-5 and occludin, the synaptic proteins SYP and PSD95, and the activity of astrocytes within 120 h during pMCAO-induced BBB leakage.

The BBB is the gatekeeper of the CNS, where it acts as the immunological, transport, metabolic, and physical boundary that separates the brain from systemic circulation [16–19]. Disruption of the BBB is one of the first events after ischemic stroke. After ischemia, the integrity of the BBB is destroyed, leading to the infiltration of inflammatory cells and high-molecular-weight proteins into the brain, resulting in cerebral infarction and edema [20]. Peripheral injection of Evans blue (961 Da) is the classic method for measuring BBB leakage. Evans blue binds to serum albumin, thereby becoming a tracer for high-molecular-weight molecules that can penetrate the brain after the BBB is damaged [21].

Here, we show that the integrity of the BBB in the ischemia-affected hemisphere was significantly impaired from 4 to 120 h after ischemia. BBB permeability was highest at 8 and 120 h after ischemia. This result is interesting because it is somewhat similar to a study using an

ischemia–reperfusion model [9]. It is possible that the first peak of BBB permeability is during an acute hyperemia period resulting from a quick elevation in cerebral blood flow [22, 23]. We speculate that the second peak of BBB permeability is due to the appearance of inflammatory cytokines released by microglia and astrocytes; these cytokines then damage cerebral microvessel endothelial cells [24–26]. Compared to the ischemia–reperfusion model, the permanent ischemia model causes more severe injury. The peak of Evans blue content in brain tissue is 0.94 $\mu\text{g/g}$ in our study, whereas in the ischemia–reperfusion model the peak content is just 0.4 $\mu\text{g/g}$ [9].

TJs are one form of intercellular adhesion structures and are responsible for sealing the intervening space in epithelial and endothelial cell sheets [27]. The TJ proteins claudin-5 and occludin perform a vital role as structural and functional components of TJs. Claudin-5, a transmembrane TJ protein, has been identified in BBB endothelial cells and directly determines BBB integrity [16, 28]. Regulation of claudin-5 activity by PKA and Rho kinases has been characterized in the process of TJ permeability [29]. Increased expression of claudin-5 increases the small-molecule paracellular permeability at the BBB. Occludin is highly expressed in brain microvascular endothelial cells, and it is detected consistently along cell margins. Although several experiments have shown that occludin is not correlated with the formation of TJs, occludin is still a critical regulatory protein of BBB function [13]. The presence of occludin in the cell membrane mediates TJ responses to inflammation and acute rCBF changes [30]. Regulatory actions of occludin are influenced by PKC, connexin-26, and PI3K (p85) [31]. Based on the key roles of claudin-5 and occludin in pMCAO-induced BBB leakage, we used western blotting to assess the changes over time in the presence of these two TJ proteins in ischemic penumbra tissues of the brain within 120 h after ischemia. Here, we demonstrate that the expression of these proteins is significantly decreased at 8, 12, and 120 h after pMCAO, which is consistent with the two peaks of Evans blue leakage. The data suggest that the reduction of claudin-5 and occludin in ischemic hemisphere contributes to impairment of BBB integrity over time following focal ischemia. In this study, TJ proteins are up-regulated at 72 h and EB leakage is suppressed at this same time point. In cerebral ischemia models, the time point 72 h belongs to the subacute phase. Compared with 24 and 48 h after pMCAO, the motor function of rats shows slight recovery at 72 h in rotarod test [32]. The levels of transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and of heme oxygenase-1 (HO-1) peak at 24 h and decrease at 72 h after ischemia, and NF- κB is also down-regulated at 72 h [33, 34]. In addition, the serum level of the proinflammatory factor TNF- α is decreased, while that of the

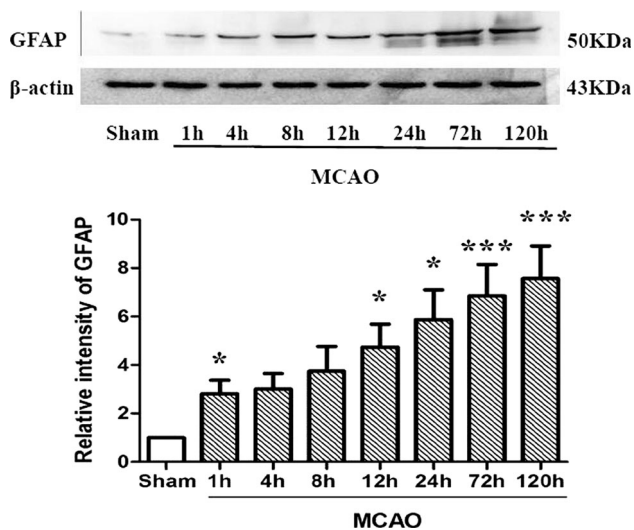


Fig. 5 The expression of GFAP in penumbra at different time points after MCAO. Expression level of GFAP at 12, 24, 72, and 120 h after ischemia were significantly increased compared to the sham group. Data represent mean \pm SEM, $n = 6$. * $p < 0.05$, *** $p < 0.001$ vs. sham group

anti-inflammatory cytokine IL-10 is increased at 72 h after ischemia [34]. Therefore, we speculate that transcription factors regulate the levels of aquaporins, TJ proteins and inflammatory cytokines, further inducing a recovery of BBB integrity. However, unlike the results for EB leakage and TJ proteins, the percentage of the infarct volume increases with time after ischemia, and the expression levels of synaptic proteins decrease. Because the infarct core expands quickly, therapeutic interventions are not completely effective if blood flow is not fully restored within the revival time after ischemia. Endothelial cells, along with other supporting cells such as neurons, pericytes, astrocytes, and microglia, determine the integrity of the BBB and are collectively known as the neurovascular unit [35]. The synthesis of GFAP by mature astrocytes can be stimulated by a large number of physical and chemical insults, and GFAP up-regulation is one of the most important features of astrogliosis [36, 37]. Our data showed that the expression of GFAP was markedly increased in a time-dependent manner, suggesting that astrocyte activation contributes to the impairment of BBB integrity after focal ischemia.

In conclusion, our data provide a time-course of BBB leakage and TJ protein changes within 120 h after permanent focal ischemia in a rat model. These results are helpful for understanding the characteristics of pMCAO and support the translational potential of animal stroke models.

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Compliance with ethical standards

Ethical approval All animal procedures performed in this study were approved by the Institute for Experimental Animals at Shenyang Pharmaceutical University (Permit Number: SYPU-IACUC-C2015-0831-203).

Conflict of interest The authors declare that they have no conflicts of interest.

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