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Rho kinase

A new target for treatment of cerebral ischemia/reperfusion injury★

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Abstract
Rho kinase inhibitor fasudil hydrochloride has been shown to reduce cerebral vasospasm, to inhibit inflammation and apoptosis and to promote the recovery of neurological function. However, the effect of fasudil hydrochloride on claudin-5 protein expression has not been reported after cerebral ischemia/reperfusion. Therefore, this study sought to explore the effects of fasudil hydrochloride on blood-brain barrier permeability, growth-associated protein-43 and claudin-5 protein expression, and to further understand the neuroprotective effect of fasudil hydrochloride. A focal cerebral ischemia/reperfusion model was established using the intraluminal suture technique. Fasudil hydrochloride (15 mg/kg) was intraperitoneally injected once a day. Neurological deficit was evaluated using Longa's method. Changes in permeability of blood-brain barrier were measured using Evans blue. Changes in RhoA, growth-associated protein-43 and claudin-5 protein expression were detected using immunohistochemistry and western blotting. Results revealed that fasudil hydrochloride noticeably contributed to the recovery of neurological function, improved the function of blood-brain barrier, inhibited RhoA protein expression, and upregulated growth-associated protein-43 and claudin-5 protein expression following cerebral ischemia/reperfusion. Results indicated that Rho kinase exhibits a certain effect on neurovascular damage following cerebral ischemia/reperfusion. Intervention targeted Rho kinase might be a new therapeutic target in the treatment of cerebral ischemia/reperfusion.

Key Words
neural regeneration; brain injury; cerebral ischemia; Rho kinase; fasudil hydrochloride; RhoA; growth-associated protein-43; claudin-5; neurovascular unit; blood-brain barrier; grants-supported paper; neuroregeneration

Research Highlights
(1) This study sought to analyze the relationship of Rho signaling pathway changes and cerebral ischemia/reperfusion injury, and found that claudin-5 expression gradually decreased in brain tissues with increasing time.
(2) Rho kinase inhibitor fasudil hydrochloride upregulated claudin-5 protein expression, lessened blood-brain barrier permeability, reduced RhoA protein expression, increased growth-associated protein-43 protein expression, and promoted the recovery of neurological function in brain tissues following cerebral ischemia/reperfusion injury.

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INTRODUCTION

The Rho subfamily includes the isoforms RhoA, RhoB, and RhoC, with RhoA mainly localized to neurons[1]. Rho kinase is a kind of serine/threonine protein kinase, can be activated by Rho, and is the downstream substrate of Rho A[1]. Numerous studies have confirmed that the Rho kinase signaling pathway participates in cerebral vasospasm after subarachnoid hemorrhage[2-3], neural cell apoptosis[4-5], inflammation[6-7], cerebral blood flow regulation[8-9], brain microvessel function regulation[9] and nerve axon growth[10-12]. These findings suggest that Rho kinase plays an important role in cerebral ischemia/ reperfusion.

Fasudil hydrochloride is a potent Rho-kinase inhibitor[13]. Studies have demonstrated that the Rho kinase inhibitors Y-27632 and H-1152 promote axon growth of nerve cells by suppressing the RhoA/Rho kinase pathway[14-17]. Moreover, erythropoietin promotes the regeneration of nerve axons, which is associated with its inhibitory effect on the RhoA/Rho kinase pathway[18]. Rho kinase inhibitor fasudil can lessen secondary inflammatory injury induced by experimental spinal cord injury[19]. Ischemic spinal cord injury can be protected by the reduction of spinal cord ventral horn cell leakage and by the increase of inducible nitric oxide synthase expression[20]. Couch *et al* [21] observed that fasudil increased dendrite budding in transgenic mice, and contributed to the growth of pyramidal neuron dendrites in the CA1 region. It is evident that Rho plays a key role in mediating the effect of inhibitory signals on axonal regeneration[22]. The inhibition of the Rho-kinase signaling pathway can prevent inhibitory molecules in the myelin sheath and contribute to the regeneration of axons and dendrites following degeneration.

Growth-associated protein-43, a nervous tissue specific protein which is highly expressed in neurons during development and nerve regeneration, is involved in axon growth and synaptic formation[23-26]. High expression of growth-associated protein-43 is considered as a marker for neuronal development, growth, regeneration and synaptic reconstruction and plasticity[23-26].

Neurovascular unit damage and protection following cerebral ischemia/reperfusion is a focus area of study in stroke research. The blood-brain barrier is part of the neurovascular unit. The changes in blood-brain barrier permeability is an essential pathological process in cerebral ischemia/reperfusion injury, and could lead to cerebral edema and hemorrhagic transformation, affecting the recovery of neurological function[27-29]. Claudin-5 is a main transmembrane protein of the blood-brain barrier. This protein is necessary for the formation of tight junctions found in the blood-brain barrier, and is an important regulatory factor for vascular endothelial cell permeability[30]. The increase in blood-brain barrier permeability might be associated with decreased claudin-5 expression during cerebral ischemia/reperfusion[31-32].

It remains unclear whether changes in the Rho signaling pathway affect claudin-5 following cerebral ischemia/reperfusion, alter blood-brain barrier permeability, or are associated with growth-associated protein-43. Thus, the present study was designed to investigate the effects of the Rho kinase inhibitor, fasudil hydrochloride, on blood-brain barrier permeability and changes in RhoA, growth-associated protein-43 and claudin-5 protein expression in a rat model of focal cerebral ischemia/reperfusion injury. The promoting effects of fasudil hydrochloride on the recovery of neurological function, and the effects of Rho kinase on cerebral ischemia/reperfusion were also explored.

RESULTS

Quantitative analysis of experimental animals

Of 110 included rats, 6 rats were allocated to the sham operation group, and the remaining 104 rats were used to establish a model of right cerebral ischemia/ reperfusion injury using middle cerebral artery occlusion. Eight rats were not included in the final analysis. Two were affected by subarachnoid hemorrhage, one was not conscious at 3 hours following anesthesia, one died after the operation, two scored 0 in the neurological deficit scale; and two scored 4 points in the neurological deficit scale.

A total of 96 rats were included in the final analysis. Seventy-two rats remained and were equally and randomly assigned to the cerebral ischemia control group, saline group and fasudil hydrochloride group, and were used for immunohistochemistry and western blotting. The remaining 24 rats were equally and randomly divided into the saline and fasudil hydrochloride groups, and used for the determination of blood-brain barrier permeability. The fasudil hydrochloride group was injected with fasudil hydrochloride, and the saline group was treated with an equal volume of saline.

Fasudil hydrochloride promoted the recovery of neurological function in rats with cerebral ischemia/reperfusion injury

Longa's score revealed that the neurological function in the fasudil hydrochloride group was significantly better than that in the cerebral ischemia control group and saline group at 1, 3, and 7 days following cerebral ischemia/reperfusion ($P < 0.05$ or $P < 0.01$; Figure 1).

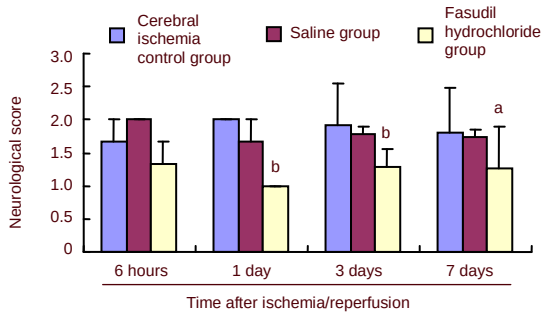

Figure 1 Fasudil hydrochloride improved the neurological function in rats with cerebral ischemia/reperfusion injury.

Rat behavioral disturbance was scored according to Longa's method. A low score indicates good neurological function. Results are expressed as mean ± SD ($n = 6$). [a]$P < 0.05$, [b]$P < 0.01$, *vs*. cerebral ischemia control and saline groups (rank-sum test). Rats in the sham operation group did not experience neurological deficit, and received a score of 0.

Fasudil hydrochloride improved blood-brain barrier permeability in rats with cerebral ischemia/reperfusion injury

At 12 hours following cerebral ischemia/reperfusion, no significant difference in Evans blue content was detected in brain tissue between the fasudil hydrochloride group and saline group ($P < 0.05$). At 24 hours, the Evans blue content in the fasudil hydrochloride group was significantly lower compared with the saline group ($P < 0.01$; Figure 2).

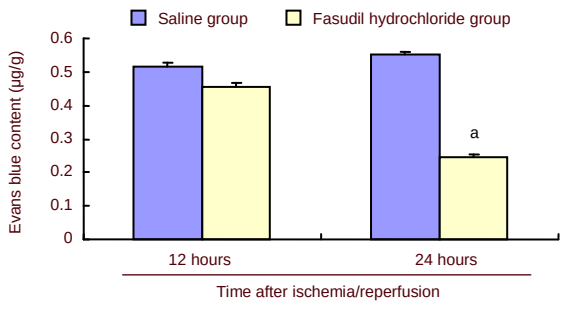

Figure 2 Fasudil hydrochloride improved blood-brain barrier permeability in rats with cerebral ischemia/reperfusion injury.

Blood-brain barrier permeability was measured using the Evans blue-methanamide method. Results are expressed as mean ± SD ($n = 6$). [a]$P < 0.01$, *vs*. saline group (*t*-test).

Fasudil hydrochloride decreased RhoA protein expression in brain tissue following cerebral ischemia/reperfusion

Western blotting revealed that RhoA expression was lower in the fasudil hydrochloride group at 1 day following reperfusion compared with the cerebral ischemia control and saline groups ($P < 0.05$). RhoA expression was significantly diminished at 3 and 7 days following reperfusion ($P < 0.01$; Figure 3). Immunohistochemical results are displayed in Figure 4. Weak RhoA immunoreactivity was visible in the sham operation group. At 6 hours following ischemia/reperfusion, RhoA immunoreactivity density and intensity were similar in various groups, with the presence of slightly light brown staining. With increasing time, the density of RhoA-immunoreactive cells increased, and staining intensity became dark in the cerebral ischemia control and saline groups, but the density of RhoA-immunoreactive cells reduced, and staining intensity became light in the fasudil hydrochloride group.

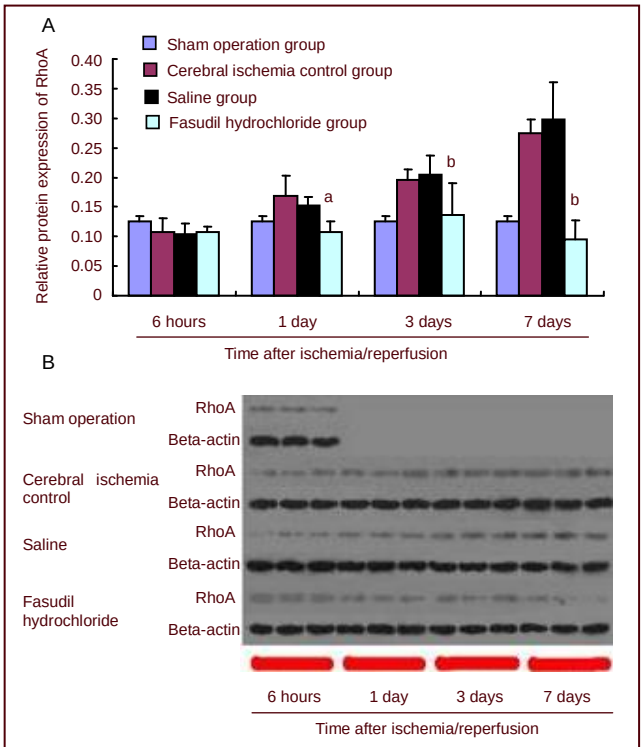

Figure 3 Effects of fasudil hydrochloride on RhoA protein expression in brain tissue surrounding the ischemic foci from rats with cerebral ischemia/reperfusion injury.

RhoA protein expression was measured using western blotting, and analyzed using Gel pro4.0 software. (A) Quantitative analysis of RhoA protein expression: data are the ratio of cumulative absorbance of RhoA protein to beta-actin, and are expressed as mean ± SD ($n = 3$). [a]$P < 0.05$, [b]$P < 0.01$, *vs*. cerebral ischemia control and saline groups (one-way analysis of variance). (B) Western blotting showing RhoA expression in various conditions.

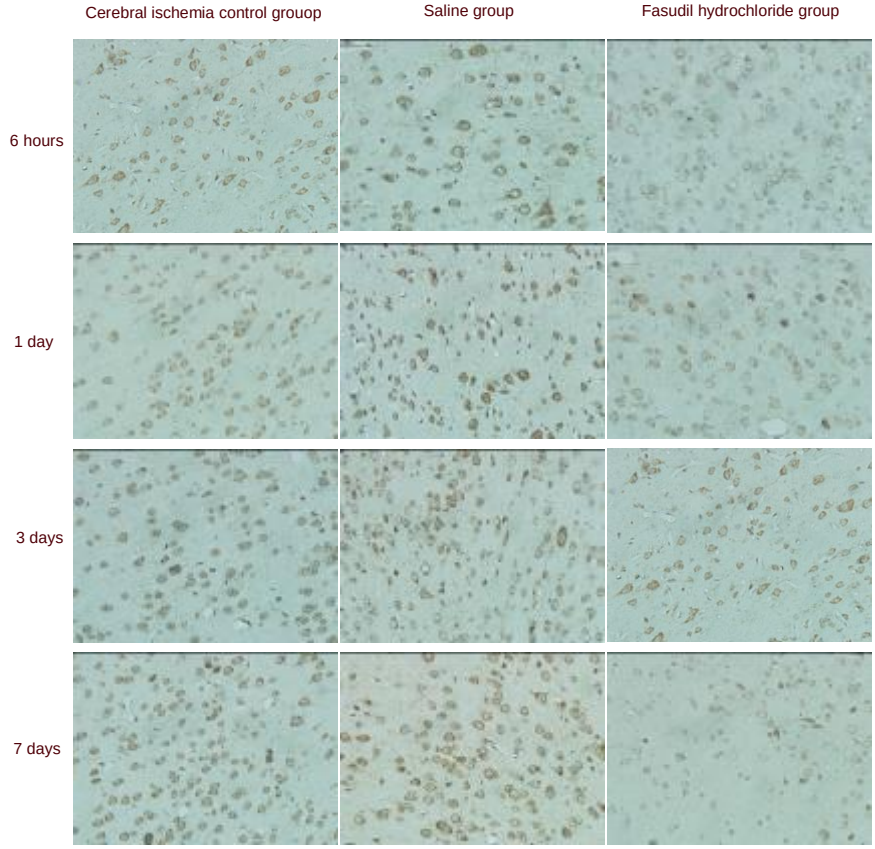

Figure 4 Effects of fasudil hydrochloride on RhoA immunoreactivity in brain tissues surrounding the ischemic foci of rats with cerebral ischemia/reperfusion injury (immunohistochemistry, × 40).

RhoA-immunoreactive cells were darkly stained brown, and mainly expressed in neuronal membranes. At 6 hours following ischemia/reperfusion, RhoA immunoreactivity density and intensity were similar in the cerebral ischemia control, saline and fasudil hydrochloride groups, with the presence of slightly light brown staining. With increasing time, the density of RhoA-immunoreactive cells increased, and staining intensity became dark in the cerebral ischemia control and saline groups, but the density of RhoA-positive cells reduced, and staining intensity became lighter in the fasudil hydrochloride group.

Fasudil hydrochloride increased growth-associated protein-43 protein expression in brain tissues after cerebral ischemia/reperfusion

Western blotting results demonstrated that growth-associated protein-43 protein expression was higher in the fasudil hydrochloride group compared with the cerebral ischemia control and saline groups at 1, 3 and 7 days following reperfusion ($P < 0.01$). With increasing time, growth-associated protein-43 protein expression significantly increased (Figure 5). Immunohistochemical results are displayed in Figure 6. A small number of growth-associated protein-43 immunoreactive cells were shown in the sham operation group. After ischemia/reperfusion, growth-associated protein-43 immunoreactivity increased in the cerebral ischemia control, saline and fasudil hydrochloride groups. The immunoreactivity of growth-associated protein-43 displayed a tendency to increase over time, especially in the fasudil hydrochloride group.

Fasudil hydrochloride upregulated claudin-5 protein expression in brain tissue of rats with cerebral ischemia/reperfusion injury

At 6 hours, 1, 3, and 7 days following ischemia/reperfusion, claudin-5 expression significantly decreased in each group ($P < 0.01$). At 3 days following reperfusion, claudin-5 expression in brain tissues significantly increased in the fasudil hydrochloride group ($P < 0.05$), with the increase being more significant at 7 days ($P < 0.01$; Figure 7).

DISCUSSION

This study observed that RhoA, growth-associated protein-43 and claudin-5 protein expression altered following cerebral ischemia/reperfusion. RhoA was inhibited, but growth-associated protein-43 and claudin-5 protein expression increased.

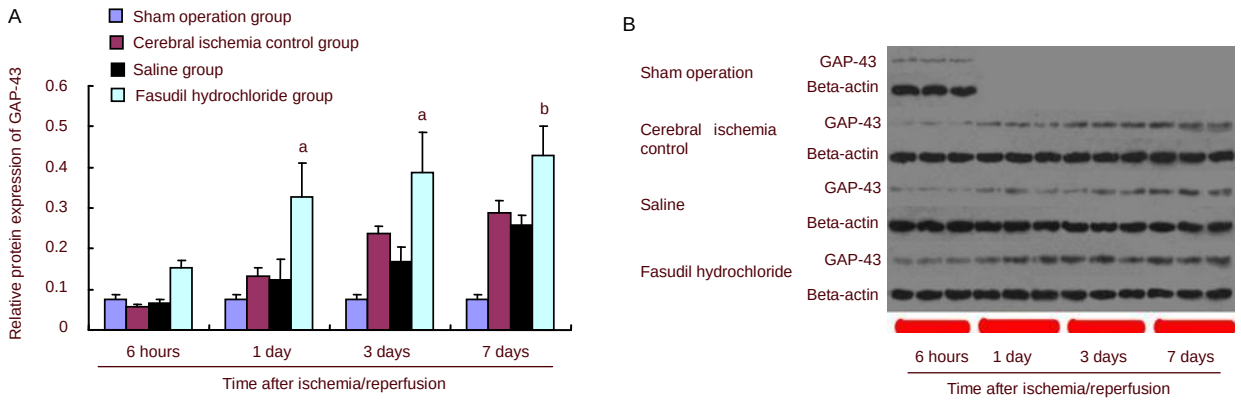

Figure 5 Effects of fasudil hydrochloride on growth-associated protein-43 (GAP-43) protein expression in brain tissues surrounding the ischemic foci of rats with cerebral ischemia/reperfusion injury.

GAP-43 protein expression was measured by western blotting, and analyzed using Gel pro4.0 software. (A) Results of quantitative detection of GAP-43 protein expression: data are the ratio of accumulative absorbance of GAP-43 to beta-actin, and expressed as mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. cerebral ischemia control and saline groups (one-way analysis of variance). (B) Western blot bands of GAP-43 and beta-actin expression in the indicated groups.

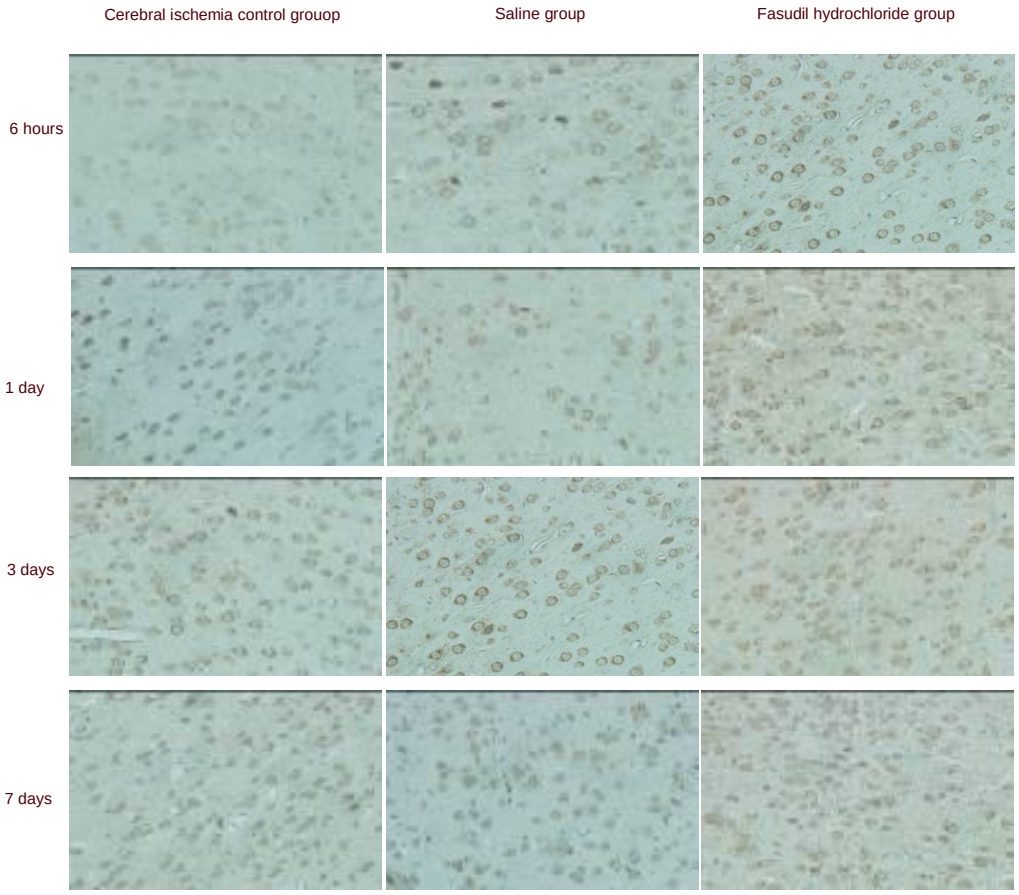

Figure 6 Effects of fasudil hydrochloride on growth-associated protein-43 immunoreactivity in brain tissue surrounding the ischemic foci of rats with cerebral ischemia/reperfusion injury (immunohistochemistry, × 40).

Growth-associated protein-43-immunoreactive cells were darkly stained brown, and mainly expressed in neuronal membranes and cytoplasm. With increasing time, the density of growth-associated protein-43-immunoreactive cells increased, and staining intensity became darker in the cerebral ischemia control, saline and especially in the fasudil hydrochloride group.

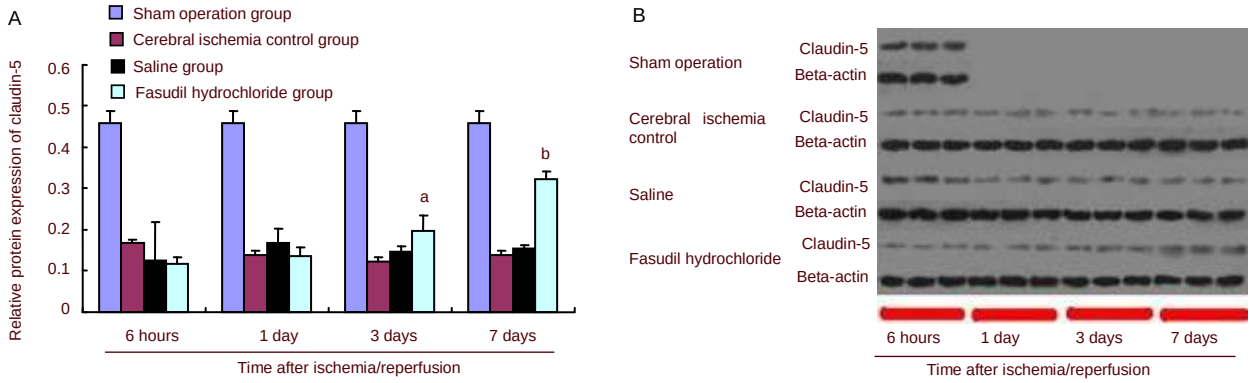

Figure 7 Effects of fasudil hydrochloride on claudin-5 protein expression in brain tissue surrounding the ischemic foci of rats with cerebral ischemia/reperfusion injury.

Claudin-5 protein expression was measured by western blot analysis, and analyzed using Gel pro4.0 software. (A) Results of quantitative detection of claudin-5 protein expression: data are the ratio of cumulative absorbance of claudin-5 protein and beta-actin, and expressed as mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. cerebral ischemia control and saline groups (one-way analysis of variance). (B) Western blot bands of claudin-5 and beta-actin expression in the indicated groups.

Fasudil hydrochloride lessened the damage of blood-brain barrier permeability and promoted the recovery of neurological function by suppressing RhoA expression and by increasing growth-associated protein-43 and claudin-5 expression. Using immunohistochemistry and western blotting, RhoA expression gradually increased, with increasing time, in the cerebral ischemia control and saline groups. Brabeck *et al* [33] observed RhoA and RhoB expression upregulated at 2–10 days at the lesion site in humans following focal ischemia for 4–38 consecutive months. Fasudil hydrochloride significantly reduced RhoA expression at 3 and 7 days following reperfusion. Moreover, neurological deficit scores obviously diminished in the fasudil hydrochloride group. Multicenter clinical trials confirmed that fasudil hydrochloride noticeably promoted the recovery of neurological function at 2 weeks following stroke and improved functional prognosis at 1 month[34]. The Rho kinase inhibitor, Y-27632, contributed to the recovery of learning and spatial memory abilities in rats at 30 days following cerebral ischemia[35]. These results suggested that Rho kinase and Rho kinase inhibitors play an important role in cerebral ischemia. A Rho kinase inhibitor could promote the recovery of neurological function after cerebral ischemia, with its mechanisms most likely being associated with the inhibition multiple reactions in the Nogo-NgR-P75NTR-Rho/Rho-kinase signaling pathways[36-37]. Moreover, fasudil relieved the damage of ischemia/hypoxia reperfusion by suppressing the interaction of inflammatory leukocytes and endothelial cells[38] and by inhibiting the inflammatory reaction of microglia[39]. Fasudil exerted neuroprotective effects by improving endothelial cell function[40]. Fasudil diminished neural cell apoptosis by Akt activation and PTEN inhibition[41]. Fasudil contributed to neural regeneration by stimulating astrocytes to produce colony-stimulating factors[42]. Fasudil could directly exert protective effects on neurons against ischemic injury[43]. Taken together, the Rho-kinase signaling pathway is possibly a key target for the treatment of cerebral ischemia/reperfusion injury.

This study observed that the Evans blue content was significantly decreased in the fasudil hydrochloride group than that in the saline group at 24 hours following ischemia/reperfusion. Liu *et al* [10] observed that Rho kinase inhibitor could diminish blood-brain barrier permeability, and lessen cerebral ischemia injury by lightening vasogenic brain edema. It is evident that the blood-brain barrier is part of the composition of the neurovascular unit. Intercellular tight junctions form the basis of creating the blood-brain barrier. Claudin is an important protein in tight junction formation, and exerts a crucial effect on the changes in blood-brain barrier permeability[44]. Claudin-1, claudin-3, claudin-5, and claudin-12 expression has been shown in cerebral vascular endothelial cells. Claudin-5 is a transmembrane protein reported to be primarily present in tight junctions of endothelia[45]. Fontijn *et al* [30] reported that claudin-5 is an important regulatory factor for the regulation of cerebral vascular endothelial cell permeability. Therefore, claudin-5 probably plays an essential role in blood-brain barrier permeability.

Western blotting results confirmed that fasudil hydrochloride increased claudin-5 expression in brain

tissue at various time points following cerebral ischemia/reperfusion. Yang and Rosenberg[27] verified that matrix metalloproteinase activation mediated claudin-5 damage following cerebral ischemia. Zehendner *et al* [46] verified that tight junction proteins in the neurovascular unit were damaged in early cerebral ischemia, and Caspase-3 activation led to claudin-5 damage. Liu *et al* [47] found that Caveolin-1 mediated claudin-5 redistribution in early cerebral ischemia. It is presumed that the mechanism of action of fasudil hydrochloride (upregulation of claudin-5 protein expression) is probably associated with the inhibition of matrix metalloproteinases, Caspase-3 or Caveolin-1, which protects the integrity of the neurovascular unit. The precise mechanisms of this compound, however, still require further investigation.

Immunohistochemistry and western blotting results demonstrated that growth-associated protein-43 protein expression increased in brain tissues surrounding the ischemic foci of rats at 3 days following cerebral ischemia/reperfusion injury. Growth-associated protein-43 protein expression was significantly higher in rats in the fasudil hydrochloride group than that in rats with cerebral ischemia/reperfusion injury at 6 hours and 1 day following ischemia/reperfusion, and this tendency lasted until 7 days following reperfusion. These results indicate that cerebral ischemia induced neurogenesis and synaptic formation, and that brain tissue has limited self-repairing capability with prolonged time following ischemia/reperfusion. Growth-associated protein-43 protein expression increased with early use of fasudil hydrochloride, and lasted until 7 days following treatment. Thus, this Rho kinase inhibitor upregulated protein expression of growth-associated protein-43 in middle cerebral artery occlusion rats following cerebral ischemia/reperfusion. This action may be associated with growth-associated protein-43 phosphorylation[48]. The upregulation mentioned above was probably due to fasudil promoting neural regeneration and functional recovery following cerebral ischemia/reperfusion.

In summary, Rho kinase is involved in neurovascular unit damage following cerebral ischemia/reperfusion, illustrating this protein as a new target for treatment of cerebral ischemia/reperfusion injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

Experiments were performed in the Department of Neurology, Beijing Friendship Hospital, Capital Medical University, China from January 2011 to March 2012.

Materials

Animals: 110 healthy clean adult male Sprague-Dawley rats weighing 240–280 g were provided by the Experimental Animal Center of Academy of Military Medical Sciences in China (license No. SCXK (Jun) 2002-001). Rats were fasted for 12 hours before the operation, but allowed free access to water. Animal procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[49].

Drugs: fasudil hydrochloride injection, hexahydro-1-(5-isoquinolinesulfonyl)-1H-1,4-diazepine hydrochloride, molecular formula: $C_{14}H_{17}N_3O_2S{\cdot}HCl$, purchased from Tianjin Chase Sun Pharmaceutical Co., Ltd.; specifications: 2 mL:30 mg; approval No. GYZZ H20040356.

Methods

Preparation of rat models of focal cerebral ischemia/reperfusion injury

In accordance with the Bederson and Longa's method[50-51], a rat model of right middle cerebral artery occlusion ischemia/reperfusion was established. Rats were intraperitoneally anesthetized with 10% chloral hydrate (0.35 mL/100 g), and placed on an operation plate in the supine position. Their heads and limbs were fixed. After shaving and sterilization, a cervical median incision (3–4 cm long) was made. Precervical fascia and muscle were isolated with forceps, and fascia and muscle on the inside of the sternocleidomastoid were dissociated. Arterial pulses were visible. Tissues surrounding the artery were carefully dissociated, without injury to the vagus nerve. The right common carotid artery and external carotid artery were dissociated towards the direction of the head. The proximal part of the right common carotid artery and root of the external carotid artery were ligated. The pterygopalatine artery was dissociated upwards along the internal carotid artery, and ligated. Blood flow in the right common carotid artery and internal carotid artery was interrupted by a thread. A small cut was made at 0.5 cm from the proximal part of the external carotid artery, and a thread was inserted into the internal carotid artery along the external carotid artery. After blood flow was restored, the thread was inserted about 1.8 cm until resistance was felt. Blood flow in the middle cerebral artery was blocked when the initial end

of the middle cerebral artery and proximal part of anterior cerebral artery were occluded by the thread. Rectal temperature was monitored during the operation, and maintained at 37°C with a light. Rats were housed in individual cages to prevent asphyxia. Reperfusion was conducted at various time points after embolism. In the sham operation group, right common carotid artery, external carotid artery and internal carotid artery were only dissociated, without inserting any thread.

Neurological deficit scores

Rats were conscious at 2 hours after anesthesia. Neurological deficit was assessed during reperfusion in accordance with the Longa's method[50-51]. A modified scale was used for neurologic assessment: 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the lateral side; 3, falling to the contralateral side; and 4, no spontaneous walking, with a loss of consciousness. In the sham operation group, no deficit was detected, with a score of 0. Ischemia/reperfusion rats which scored 1–3 points were included in the experiments. After grouping, mental status was observed daily in rats with ischemia/ reperfusion injury, and neurological deficit score was recovered. The average value in each group was calculated at 6 hours, 1, 3, and 7 days following reperfusion.

Drug injection

Rats in the fasudil hydrochloride group were intraperitoneally injected with 15 mg/kg fasudil hydrochloride immediately after reperfusion, once a day. Rats in the saline group were intraperitoneally injected with an equal volume of saline, once a day. Rats in the cerebral ischemia control group and sham operation group were conventionally fed, without injection or other therapies. The rats used for immunohistochemical staining and western blotting received ischemia for 2 hours and reperfusion for 6 hours, 1, 3, and 7 days. Six rats were sacrificed in each group at various time points. Three rats from each group were subjected to immunohistochemical staining for RhoA and growth-associated protein-43, and the remaining three rats underwent western blotting for RhoA, growth-associated protein-43 and claudin-5 protein expression. At 2 hours after ischemia and 12 and 24 hours after reperfusion, six rats from each group were sacrificed for determination of blood-brain barrier permeability.

Evans blue-methanamide method for detection of blood-brain barrier permeability

In accordance with a previously published study[31], rats were injected with 2% Evans blue (2 mg/kg) *via* the femoral vein at 1 hour before sacrifice. Perfusion with saline was conducted through the left ventricle until clear liquid flowed from the right auricle. Rat brain tissue was weighed and incubated in methanamide solution (1 mL/ 100 mg) at 60°C for 24 hours, and centrifuged at 3 000 r/min for 10 minutes. Absorbance values were measured at 632 nm with a spectrophotometer (Jenway, Felsted, UK). Concentration values were obtained by comparing with a standard curve, and divided tissue weight, *i.e.*, Evans blue content, which was expressed as μg/g.

Immunohistochemical staining for RhoA and growth-associated protein-43

Brain tissues at 2 mm posterior to the optic chiasma were coronally sliced into sections, dehydrated, embedded in paraffin and sliced into 5 μm-thick sections. Sections were incubated in primary antibodies, rabbit anti-RhoA polyclonal antibody (Abcam, Cambridge, UK) and rabbit anti-growth-associated protein-43 polyclonal antibody (Abcam), with a working concentration of 1:200 at 4°C for 18 hours. This incubation was followed by secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China), with a working concentration of 1:200 at 37°C for 30 minutes. Each specimen was observed and photographed with a light microscope (Olympus, Tokyo, Japan). Three visual fields of one section from each rat were randomly selected for each index under a 40 × objective lens.

Western blotting for RhoA, growth-associated protein-43 and claudin-5 protein expression

Brain tissues (0.5 mg) surrounding the ischemic foci were homogenized to extract total protein by lysis, followed by protein concentration detection, protein degeneration, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and membrane transfer. Rabbit anti-RhoA polyclonal antibody, rabbit anti-growth- associated protein-43 polyclonal antibody, and rabbit anti-claudin-5 polyclonal antibody (Abgab, Chicago, IL, USA) were added to combine with target protein, with a working concentration of 1:300 at 4°C for 18 hours. The membranes were then incubated with secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG with a working concentration of 1:30 000 at 37°C for 1 hour on a shaking bed. Subsequently, specimens were incubated in primary antibody mouse anti-beta-actin antibody (CST, USA) with a working concentration of 1: 1 000 at 4°C for 18 hours. Membranes were then incubated with a secondary antibody goat anti-mouse IgG/horseradish peroxidase (Zymed, San Diego, CA, USA) with a working concentration of 1:50 000 at 37°C for 1 hour on a shaking bed. This incubation was followed by chemiluminescence and quantitative analysis of western blotting. Positive

bands were analyzed using Gel pro 4.0 software (Media Cybernetics, Bethesda, MD, USA), and its accumulative absorbance values were measured.

Statistical analysis

All data were analyzed using SPSS 16.0 software (Chicago, IL, USA). Results were expressed as mean ± SD. Completely randomized multi-sample comparison was made using one-way analysis of variance. Two-sample comparison was performed using *t*-test. Rank-sum tests were used if heterogeneity of variance was present.

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Author contributions: Qinghong Cui collected and analyzed experimental data, and wrote the manuscript. Yongbo Zhang obtained funding, provided technical support and guidance, wrote and revised the manuscript. Hui Chen collected and analyzed experimental data. Jimei Li provided technical support and guidance. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Beijing Friendship Hospital, Capital Medical University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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