Original Article
Limb remote ischemic preconditioning protects against cerebral ischemia through down-regulation of aquaporin-4

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Abstract: Objective: Limb remote ischemic preconditioning (LRP) represents a new strategy for stroke treatment, but its mechanism is still unclear. Here, we studied whether aquaporin-4(AQP4) was involved in the neuroprotection induced by LRP against ischemia. Method: Adult SD male rats were subjected to either LRP or sham surgery and then subsequently underwent 1 h middle cerebral artery occlusion (MCAo). Animals underwent MR T2FLAIR/DWI imaging 48 h after stroke and then neurological behavior evaluation and TTC staining were carried out. Western blot and immunofluorescence staining were used to investigate the expression of AQP4. Result: LRP improved the behavioral outcomes and reduced the infarct size. MRI results indicated that LRP ameliorated brain edema after cerebral ischemia. Western blot and immunostaining analysis showed that LRP down-regulated protein expression of AQP4. Conclusion: LRP executed protective effects against stroke and might ameliorate brain edema by inhibiting expression of AQP4.

Keywords: Limb remote ischemic preconditioning, MRI, ischemia, aquaporin-4

Introduction

Stroke is one of the leading causes of death and disability worldwide, and it is difficult to treat. Ischemic preconditioning (IPC) is an attractive strategy that makes the brain and other visceral organs relatively such as heart [1], retina [2] and skeletal muscle [3] resistant to tissue injury. More recently IPC has been shown to not only protect the targeted organ itself but interestingly other distant organs as well. This phenomenon of protection afforded to an organ subsequent to sub-lethal ischemic insults to remote parts of the body is referred to as remote preconditioning (RPC) [4]. Various combinations of the anatomic site receiving the preconditioning stimulus and that subjected to "index ischemia" have been studied, affording protection of the latter organ [4]. Many studies have demonstrated RPC can protect widespread organs including the heart [5], kidney [6], stomach [7] and lung [8]. Furthermore, RPC is currently an emerging concept for experimental stroke. As a less-invasive method, LRP, which is performed in hind limbs, is one of the

most frequent remote preconditioning methods used to protect against heart and brain ischemia [9]. The protection of LRP against stoke have been demonstrated by several studies [10-14]. LRP executed both short-term [10] and long-term [9] protective effects against stroke. Ren et al [10] compared protection of LRP with different parameters, and eventually proved that rapid preconditioning with 3 cycles of 15 minutes performed immediately before stroke played neuroprotection best. These evidence illustrated the undoubtedly value of LRP toward stroke patients in future, but not far enough. More studies are needed to explore the underlying neuroprotective mechanisms mediated by LRP.

Cerebral edema, which is triggered after an ischemic insult, is believed to be the most common cause of neurological deterioration and mortality during acute cerebral ischemia [15]. Thus, it would be an effective approach for stroke recovery by alleviating cerebral edema. For ischemic brain edema, there are two major types of brain edema: cytotoxic and vasogenic

[16], both of which can be observed with Magnetic resonance imaging (MRI) in vivo. Cytotoxic edema results from the subtle disturbance in BBB permeability, which is associated with cellular disruptions in ionic homeostasis [17]. Swelling of brain cells, especially the enlargement of astrocytic end-feet is the main feature of cytotoxic edema. Diffusion-weighted imaging (DWI), as a sequence of MRI can be used to detect cytotoxic edema [18]. Whereas the vasogenic edema formation results from a dramatic increase in BBB permeability and reflects an increase in T2-Weighted Resonance Imaging (T2WI) values [19].

AQP4 is a water-channel protein expressed strongly in the brain [20] and it is involved in the transport of water from the astrocytic/extracellular compartments into the lumen of the vessel [21]. Increasing evidence shows that AQP4 plays an essential role in the pathogenesis of cerebral edema. Nevertheless, the actual role of AQP4 playing in stroke is more complicated. In cytotoxic edema, AQP4 deletion slows the rate of water entry into brain, whereas in vasogenic edema, AQP4 deletion reduces the rate of water outflow from brain parenchyma [20]. That is, AQP4 plays a dual role in the formation and resolution of both vasogenic and cytotoxic edema. Besides, AQP4 also plays a major role in processes that unrelated to brain edema, including astrocyte migration and neuronal excitability [20]. It's inferred that regulation of AQP4 after cerebral ischemia would be a potential target for the treatment of cerebral edema.

The molecular mechanism underlying LRP-induced neuroprotection against ischemia is very intricate and still not clear, it may involve the effects of neural pathway [11], humoral factors [22] and immune system as well [23, 24]. Our previous study demonstrated that LRP reduced brain edema in stroke rat [9], in the present study, we evaluated whether LRP could reduce cerebral edema through inhibiting AQP4 expression in a transient focal cerebral ischemia rat model.

Materials and methods

Animals and groups

This study was performed in strict accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This protocol was approved by the Animal Experimental Committee of FuJian Medical University, China (Permit Number: SYXK2012--0001). All efforts were made to minimize animal suffering. Adult male Sprague-Dawley rats (250 to 330 g) were randomly assigned into 3 groups: control ischemia, LRP and sham.

Transient focal cerebral ischemia and remote preconditioning

Anesthesia was induced by 5% isoflurane and maintained with 2-3% isoflurane during surgery. Core body temperature was monitored with a rectal probe and maintained at 37°C during the entire experiment. Protocols for inducing focal ischemia and LRP are shown in **Figure 1**. Focal ischemia was induced as described previously [9] with a little modification. Animals in control ischemic group received 90 min of the exposure of the left femoral artery before stroke onset. The right common carotid artery (CCA) and the external carotid artery (ECA) were exposed via a midline neck incision and a suture was circle around each artery. The distal middle cerebral artery (MCA) was then exposed. For ischemia induction, firstly the CCA was occluded by a aneurysm clip. Then the distalend of the ECA was ligatured and cut off. A 2% heparin coated, 4-0 monofilament suture, whose tip had been rounded near a flame was inserted into the ECA and gently advanced into the internal carotid artery (ICA) until it reached the proximal anterior cerebral artery thereby occluding the opening of the middle cerebral artery. The aneurysm clip was then released immediately and the monofilament suture was extracted 1 hour later. Local cerebral blood reperfusion was detected using Laser Doppler Flowmetry (PERIMED AB, PeriFlux System 5000, Stockholm, Sweden). A minimum initial reduction of 75% in the laser Doppler reading after the MCA was occluded was considered a successful model of experimental stroke (Figure S1). Limb remote preconditioning was induced as previously described [10], the left femoral artery was separated, occluded for 15 minutes and released for another 15 minutes, and repeated for 3 cycles. Stroke was performed immediately after remote preconditioning. Sham group suffered the same surgical procedure without left femoral artery or MCA occlusion.
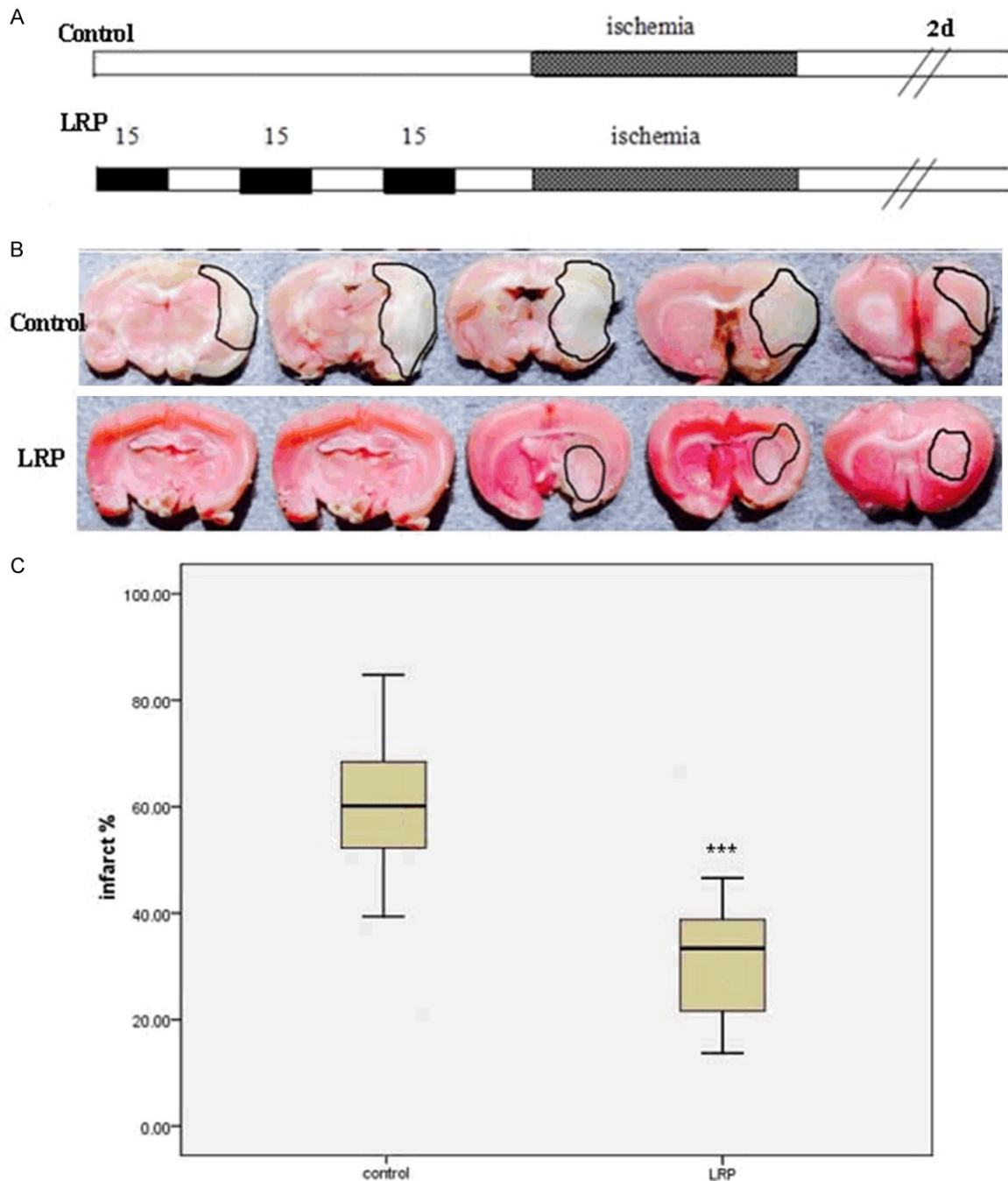
Figure 1. LRP reduced infarct size after focal ischemia. A. The diagram shows the protocol for performing ischemic preconditioning, which was conducted in the left limb by 3 cycles of 15 minutes occlusion/reperfusion. Brain ischemia was induced immediately after preconditioning. B. TTC staining from rats receiving focal ischemia with or without LRP. C. LRP reduced infarct size after stroke. Infarct size of each slice was measured and normalized to non-ischemia hemisphere as a percentage. An average value from the five levels was calculated and presented. Control ischemia, n = 5; LRP, n = 5. ***P < 0.001, vs. Control ischemia.

MRI

MR imaging was conducted with a 3.0T (GE, Discovery MR750) using a dedicated four-chan-nel phased array rat head coil assembly (WK602, Magtron Inc). Anesthesia method was the same as above. Rats were placed supinely into a plastic holder. T2-FLAIR images were

obtained using the following parameters: TR = 8450 ms, TE = 145 ms, field of view (FOV) = 100 mm, image matrix = 256 × 256, echo train length (ETL) = 32, slice thickness = 2 mm, slice interval = 0 mm, NEX = 1, Flip angle = 111°, bandwidth = 62.5 kHZ. Diffusion-weighted images were obtained using the following parameters: TR = 3000 ms, TE = 83.7 ms, field of view (FOV) = 50 mm, image matrix = 128 × 128, slice thickness = 2 mm, slice interval = 0 mm, NEX = 2, bandwidth = 166.7 kHZ and at two different 'b' values (0, 1000 s/mm) were applied. Trace-weighted apparent diffusion coefficient maps were generated. The entire MRI protocol approximately lasted for 15 min for each animal. All rats were scanned with MR imaging at 48 h after MCA occlusion. The ADC and T2WI values were measured in each slice of lesions and relevant ROI of mirror symmetry in contralateral hemisphere. The percent change was calculated using following equation: (ipsilateral value-contralateral value)/contralateral value × 100%.

Evaluation of neurological behavior

The neurologic findings were scored on all rats 48 h following MCAo on a five-point scale: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness [25].

Infarct size measurement

The rats were re-anesthetized with chloralic hydras (10%) intraperitoneally (300 mg/kg) 48 hours after stroke, perfused intracardially with 100 ml of cold 10 mM sodium phosphate buffered saline (PBS; pH 7.4). The rats were then decapitated and the brains rapidly removed and sectioned coronally at 2 mm intervals, generating a total of 5 sections. All slices were incubated in 2% 2,3,7-triphenyltetrazolium chloride (TTC) solution for 20 minutes at room temperature, fixed by immersion in 4% paraformaldehyde solution overnight. Using a computerized image analysis system Image pro plus (Version 6.0, Media Cybernetics), the area of infarction of each section was measured. Infarct size of the ischemic hemisphere was

normalized to the non-ischemic contralateral hemisphere and expressed as a percentage, and an average value from the 5 slices was presented.
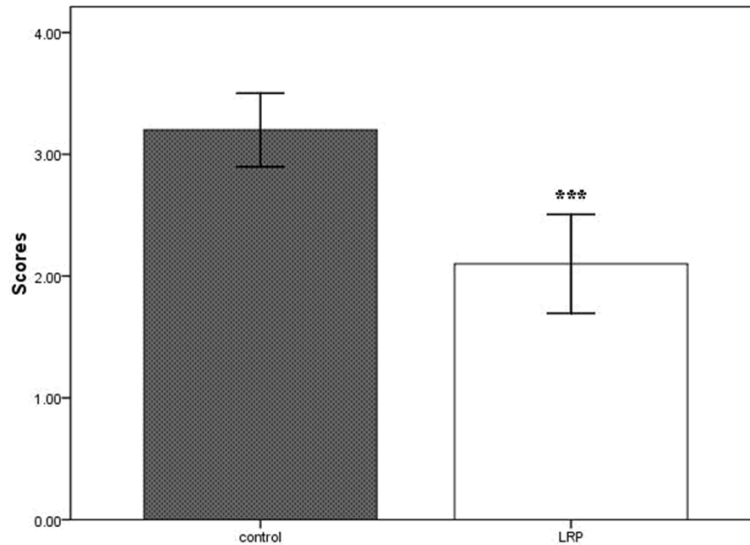
Western blot

Rats were randomly assigned into 2 subgroups, euthanized at 48 hours (n = 3/group), and rat brains were harvested for immunostaining and western blot respectively. For Western blot, samples were lysed with RIPA buffer kit (Biyuntian, China, P0013B). Extracts were homogenized and insoluble debris removed by centrifugation at 6000 g for 10 minutes at 4°C. Protein concentration in the resulting supernatants was calculated using a BCA protein assay kit according to the manufacturer's instructions (Kangweishiji, China, CW0014). Equal amounts of protein samples (13 ml) were loaded, separated using 4-15% SDS-polyacrylamide gel (Kangweishiji, China, CW-0027) electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The blot was then incubated overnight at 4°C with either a polyclonal antibody against AQP4 (1:1300; Santa Cruz Biotechnology; SC-20812) or a monoclonal antibody against β-actin (1:2000, immunoway. YT0099) in TBST (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.05% Tween 20) containing 5% skimmed milk. After washing, the membrane was incubated with horse radish peroxidase labeled antibody (1:1000, Zhongshan, China, ZB-2306) for 1 hour at room temperature. The membrane was incubated in ECL solution, and the gel image was captured using an imaging camera Bio-Rad and analyzed using gel image system (Quantity one) to estimate the integral optical density of the protein bands.

Immunofluorescence staining and confocal microscopy

Rats were trans-cardiac perfused with ice-cold PBS (pH 7.4) and 4% PFA solution. Brains were post-fixed in 4% PFA solution and cytoprotected in 20% sucrose at 4°C overnight. We stained cryostat sections (40 mm) with antibodies against AQP4 (1:100; Santa Cruz Biotechnology; SC-20812). Astrocyte cells were stained with GFAP (1:100; Santa Cruz Biotechnology, SC-56395). Vascular endothelial cells were stained with CD31 (1:100; Santa Cruz Biotechnology, SC-46694). Double staining of GFAP

LRP improved neurological behavior

LRP treated animals showed neurological scores of 2.1 ± 0.567 (n = 10), that were significantly better than ischemic control animals (3.2 ± 0.422, n = 10), (P < 0.001) (**Figure 2**).
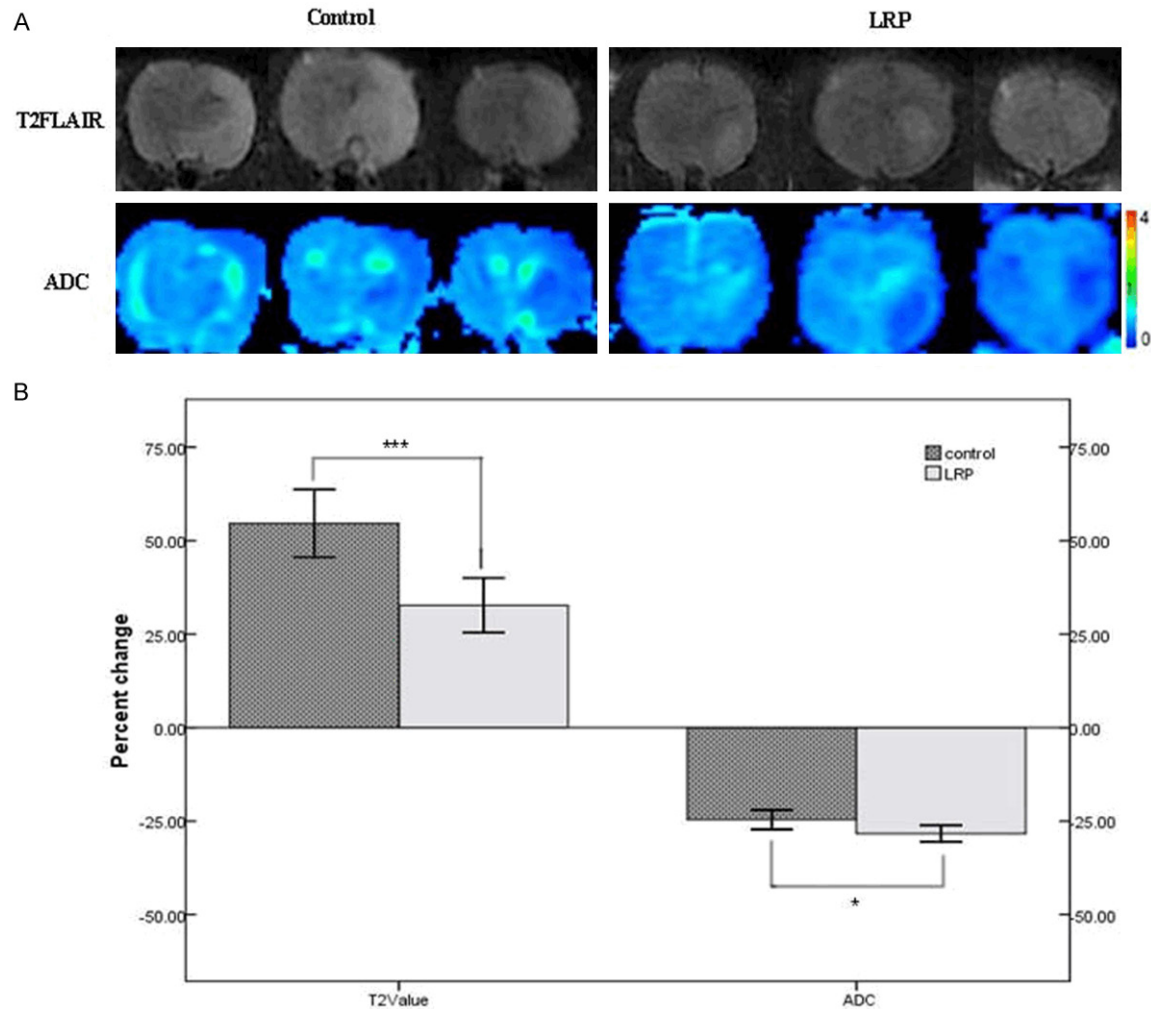
LRP ameliorated brain edema after cerebral ischemia

The lesion size of LRP group was much smaller than control group (**Figure 3A**), which was correspond with TTC result. In vascular cerebral edema stage (2 d after experimental stroke), the T2 values of the ipsilateral hemisphere were increased in both LRP group and ischemia control group. T2 values of the LRP group increased less when compared with the control group (32.712 ± 5.877% vs. 54.639 ± 7.294%, P < 0.001) (**Figure 3B**). Although ADC value means not so important for vascular edema, we did detect more decreased ADC values of ipsilateral hemisphere in LRP group than control (-28.325 ± 1.775% vs. -24.612 ± 2.085%, P < 0.05), which might offer us some messages for underlying the potential mechanism of LRP induced neuroprotection.

LRP down-regulated the protein expression of AQP4

We then investigated whether AQP4, which is involved in the transport of water from the astrocytic/extracellular compartments into the lumen of the vessel, was affected by LRP. Results by Western blot suggested that the expression of AQP4 was down-regulated by LRP compared with the control group or sham group, but there was no statistical significance of AQP4 expression between ischemia control group and sham group (**Figure 4A**, **4C**). In control group, the AQP4 expression in ipsilateral hemisphere was higher than contralateral, whereas in LRP group there was no statistical significance of AQP4 expression between the two sides (**Figure 4B**, **4D**). There was no statistical significance of AQP4 expression in contra-

Figure 2. LRP improved neurological behavior. 48 h following MCAo, the neurologic findings were scored. Limb-preconditioning treated animals showed neurological scores of 2.1 ± 0.567 (n = 10), that were significantly better than ischemic control animals (3.2 ± 0.422, n = 10), ***P < 0.001, vs. control.

and AQP4, CD31 and AQP4 were performed consecutively with their primary and secondary antibodies, respectively. Fluorescent stained sections were analyzed by confocal microscopy.

Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 20.0. Data are presented as means ± SEM. One-way analysis of variance (ANOVA) followed by post hoc Fisher's LSD tests was used for within-group comparisons. Unpaired t-tests were applied for between-group comparisons of the infarct size, neurological deficits, T2 values, rADC. P < 0.05 was considered to indicate statistical significance.

Results

LRP reduced infarct size

Results of infarct size showed that limb-preconditioning with 3 cycles of 15 minutes occlusion/ reperfusion of the femoral artery significantly reduced infarct size. Animals subject to LRP showed infarct size (29.015 ± 23.619%, n = 5) that was significantly smaller than ischemia (68.042 ± 13.244%, n = 5) (P < 0.001) (**Figure 1**).

Figure 3. LRP ameliorated brain edema after cerebral ischemia. A. T2FLAIR images and ADC maps of each group at 48 hour after MACo. The lesion was smaller in LRP group. B. The percent change of T2 value and ADC value was calculated using following equation: (ipsilateral value-contralateral value)/contralateral value × 100%. T2 values of the LRP group increased less when compared with the control group, whereas the ADC values of the LRP group decreased more. *P < 0.05, vs. control. ***P < 0.001, vs. control.

lateral hemisphere between LRP group and control group although there was less level of AQP4 expression in LRP group reflected by **Figure 4E**. The percent change between two sides of both LRP and control groups were than calculated, LRP group present less percent change compared with control group (**Figure 4E**).

Discussion

In this study, we confirmed that LRP improved the behavioral outcomes and reduced the infarct size induced by ischemia, we also used MRI to observe the effectiveness of LRP on the reduction of brain edema. Western blotting

analyses and immunofluorescence were further used to confirm that LRP could down-regulate the expression of AQP4 after ischemia. Therefore, LRP might alleviate brain edema induced by ischemia by down-regulating the expression of AQP4.

Remote preconditioning has been studied for more than 18 years [26], although priming the brain prior to index ischemia and its role in neuroprotection has been investigated extensively, the ability of the brain to protect itself from ischemic injury in response to a priming ischemic insult to a distant organ, however, remains unexplored to a large extent [11]. Remote ischemic preconditioning can be explained like this:
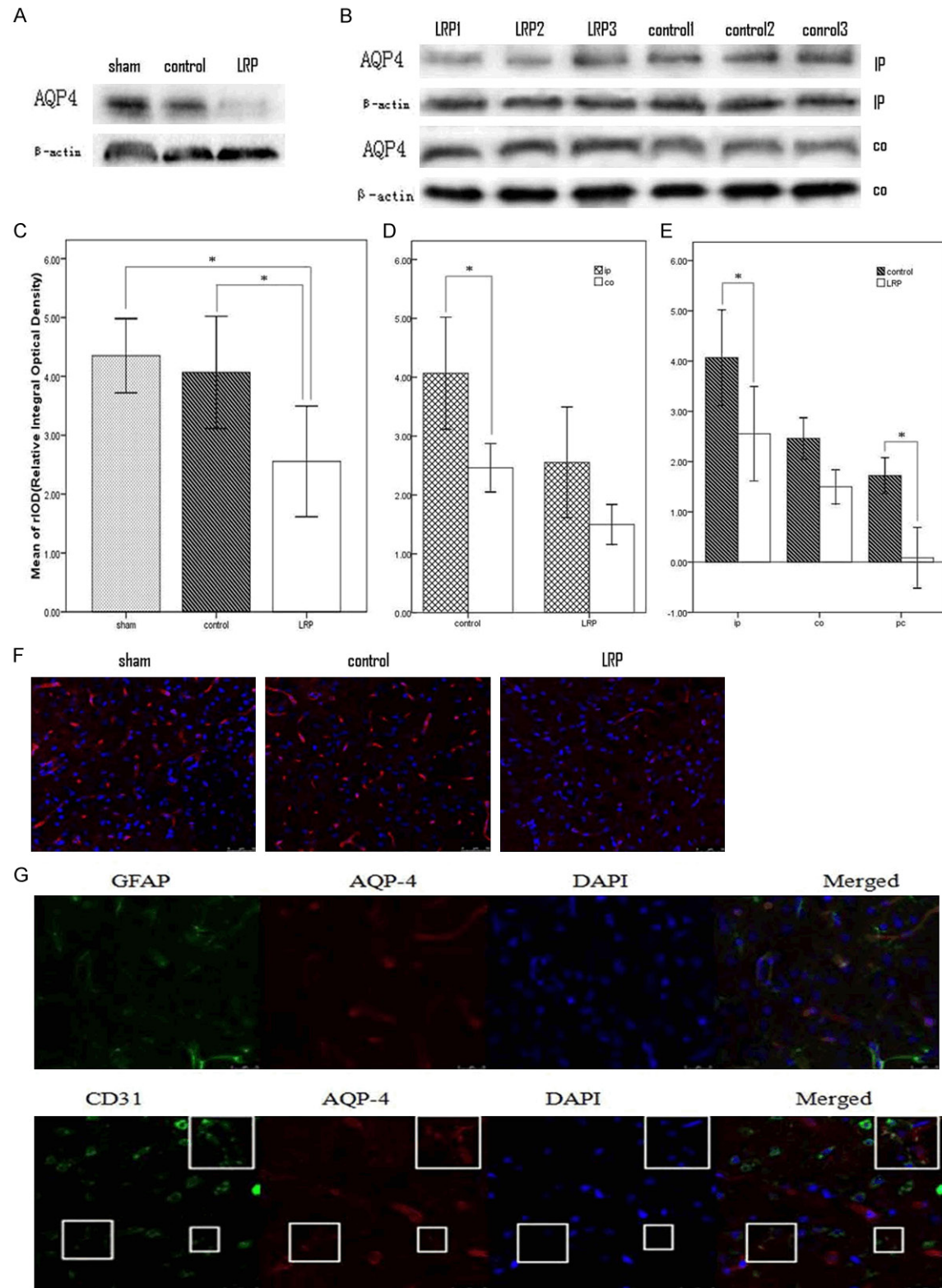
Figure 4. LRP down-regulated the protein expression of AQP4. (A) Western blot of AQP4 expression in sham, control and LRP group. (B) Western blot of AQP4 expression in bilateral hemisphere of both control and LRP group. (C-E) Semi-quantitative analysis of AQP4 expression upon Western blot, rIOD was calculated as AQP4 IOD/β-actin IOD. Results suggested that the expression of AQP4 was down-regulated by LRP compared with the control group or sham group, but there was no statistical significance of AQP4 expression between ischemia control group and sham group

(A, C). In control group, the AQP4 expression in ipsilateral hemisphere was higher than contralateral, whereas in LRP group there was no statistical significance of AQP4 expression between the two sides (B, D). There was no statistical significance of AQP4 expression in contralateral hemisphere between LRP group and control group although there was less level of AQP4 expression in LRP group reflected by (E). The percent change between two sides of both LRP and control groups were than calculated, LRP group present less percent change compared with control group (E). (F) Results from confocal microscopy indicated that AQP4 was decreased in LRP group. Scale bar, 50 µM. (G) Double staining of GFAP and AQP4, CD31 and AQP4 suggested that AQP4 was expressed in astrocyte and vascular endothelial cells. Scale bar, 25 µM. *P < 0.05.

an ischemia performed in one organ (could be kidney, limb, mesentery, skeletal muscle, liver or lung) protects against a subsequent prolonged ischemia in another distant organ. In the early experimental studies, some invasive operative procedure was used to apply the preconditioning ischemia, which was of course restricted in clinical application. Since the limb ischemia, which was introduced by Oxman et al [27], as a less invasive method of remote preconditioning stimulus was thereby widely used in experimental studies. Varied types of limb preconditioning induction were researched, for example, 3 cycles of 10 minutes occlusion of the bilateral femoral arteries followed with 10 minutes reperfusion [13], 30 minutes of a single occlusion [8], or 2/3 cycles of 5/15 minutes occlusion/reperfusion [10]. In this study, we used a LRP model according to Ren C's discovery mentioned above, and we showed remote preconditioning conducted in single hind limb ipsilateral protected against transient focal cerebral ischemia in rats based on the proof of brain injury measurement and behavioral tests, which was consistent with previous researches [10-14].

What's more, as shown in **Figure 3**, LRP ameliorated brain edema with smaller infarct size and less increased T2 values when compared with the control group, whereas the ADC values of the LRP group decreased more. In ischemic stroke, edema is commonly observed within the first week, reaching a peak 24-72 h following the ischemic event [28], it can be differentiated pathophysiologically into an early cytotoxic form, arising a few minutes following ischemia and a later vasogenic type appearing as a result of blood-brain barrier (BBB) breakdown after around 4-6 h [29]. As mentioned above, both of these two edema types can be observed with Magnetic resonance imaging (MRI) in vivo. In stroke, the apparent diffusion coefficient (ADC), as the quantitative diffusion-weighted imaging parameter, decreases within minutes and then T2 slowly increases within 24 h to days, by

which time the ADC has normalized [30]. Under pathological conditions, decreased ADC values are thought to reflect decreased extracellular space due to cellular swelling whereas the computed T2 value is representative of water content within brain tissues where increased T2 values correspond to late water accumulation [31]. In current study, we found that T2 values of the LRP group increased less when compared with the control group, it meant LRP ameliorated brain vasogenic edema efficiently. What's more, we found the ADC values of the LRP group decreased more than control group, as we mentioned above, the ADC should be normalized accompanied by T2 slowly increasing within 24 h to days after stroke. Therefore, it not only further confirmed LRP could ameliorate brain edema but also implied LRP might delay the development of brain edema that still need to be further verified.

Western blotting analyses and immunofluorescence showed that LRP could decrease the expression of AQP4 after ischemia. Aquaporin-4 (AQP4) is a hydro-selective membrane transport protein which is expressed in glial cells of the brain particularly at the borders between the brain parenchyma and major fluid compartments [17]. It is predominantly localized in astrocyte perivascular end-feet and glial limiting membranes, at the border between the brain parenchyma and subarachnoid CSF and beneath the ependyma bordering the brain parenchyma and ventricular CSF [20, 32]. AQP4 plays a key role in brain water balance in central plasma osmolarity and cerebral edema formation and regression in pathological disease [33]. Our results showed that the expression of AQP4 was down-regulated by LRP compared with the control group or sham group, what's more, within LRP group, there was no statistical significance of AQP4 expression between the two cerebral hemispheres, which meant LRP could make the expression of AQP4 in ipsilateral hemisphere normalize. This might be one of the reasons that account for the more

decreased ADC values in LRP group that we detected above, actually, it was reported that the reduction in ADC values was correlated with a decrease in AQP4 to some extent [21, 30]. On the other hand, we found the AQP4 expression in ipsilateral hemisphere was higher than contralateral in control group, but there was no statistical significance of AQP4 expression between ischemia control group and sham group. Actually, the spatiotemporal up-regulation of AQP4 is multi-factorial and dependent on differences between stroke model and type, species and age, and severity of the insult [29], so we insist the comparison between bilateral in one group is more reliable. The expression of AQP4 after stroke changes over time. In a transient MCA occlusion model, AQP4 expression is rapidly upregulated in astrocyte end-feet in contact with blood vessels, peaking at 1 h after stroke onset, and a second peak of which has been observed at 48 h that correlated with brain vasogenic edema [30, 34, 35], but the early increased AQP4 expression is not observed in models of more severe stroke, such as permanent occlusion of the distal branch of the MCA [36]. Thereby, in order to obtain stable data, we choose 48 h after stroke for detection. It is of interest that AQP4 mediates bidirectional water flux, facilitating water influx in the evolution of cytotoxic edema and water efflux during edema elimination [20]. In cytotoxic edema, AQP4 channel limits the rate of brain water accumulation by attenuating water flux from the vasculature into the brain parenchyma in the presence of an intact BBB [37, 38], whereas in vasogenic edema, it plays an opposing role. Its increased expression at astrocyte end-feet allows for polarization of function such that influx in one domain is associated with efflux in another [39]. Besides, AQP4 is also found to play a major role in processes unrelated to brain edema, including astrocyte migration and neuronal excitability [20]. Anyhow, AQP4 is a critical component regulating water movement during edema formation and resolution although the role of AQP4 in ischemia is still unclear and debated, and inhibition of AQP4 up-regulation, offers a possible therapeutic avenue to minimize brain edema. It was reported that pretreatment with an AQP4 inhibitor significantly reduced the severity of cerebral edema associated with brain ischemia [40]. In a focal ischemia model produced by MCAO, it was confirmed that AQP4-deficient mice had decreased cerebral edema and

improved neurological outcomes [41]. Our findings showed that, in LRP group, AQP4 down-regulation accompanied an obvious reduction of cerebral edema.

It is well known that ischemia-induced neuronal injury is the result of complex, sometimes irreversible, mechanisms which come into play within minutes of the ischemic insult and continue to cause neuronal cell loss for days to weeks after the initial ischemic episode has passed [42]. The phenomenon of RPC has also been investigated in various models of ischemic injury and several mechanisms of protection have been proposed, including the activation of humoral and neuronal pathways [4]. But the actual mechanism through which an episode of brief ischemia and reperfusion in an organ or tissue exerts protection against a subsequent sustained insult of ischemia-reperfusion injury in a remote organ or tissue is currently unclear [43], not mention the mechanism of neuroprotection. In the present study, we found that LRP down-regulated AQP4 expression and ameliorated brain edema. AQP4 is a downstream mediator of hypoxia induced factor 1 alpha (HIF-1α) [44]. HIF-1α, as a transcription regulatory protein, has be extensively studied as a response to low oxygen conditions and thus ischemic preconditioning in brain [45, 46]. HIF-1α is widely acknowledged to play a role in ischemic tolerance in brain, although its role in brain neuroprotection following remote preconditioning has not been established [22]. It can be hypothesized that LRP may change the level of HIF-1α and thus, down-regulate the expression of AQP4. Besides, in preconditioning (PC), the exposure to a wide range of stressors activates inflammatory pathways and leads to upregulation of inflammatory molecules similarly to those induced by stroke through toll-like receptors (TLRs) and tumor necrosis factor receptor (TNFR) signaling, which can then activate nuclear factor-κB (NF-κB) resulting in upregulation of inflammatory molecules, such as TNF-α, IL-1β, CCL2, iNOS and COX-2 [24]. These inflammatory responses and gene expression returns to basal levels when the stressor subsides. However, when the subsequently severe ischemia occurs, it may reprogram inflammatory pathways to respond differently, for instance favoring the expression of anti-inflammatory cytokines IL-10, tumor growth factor-(TGF)-β and interferon-(IFN)-β that induces ischemic tolerance [24], thus the inflamma-

tory molecules mentioned above such as IL-1β and TNF-α, both of which have the ability to up-regulate the expression of AQP4 [47, 48], are thereby downregulated. Thus, further study is needed to investigate the underlying mechanism of LRP down-regulating the AQP4 expression.

Conclusions

From the results of AQP4 protein studies and brain edema detected with MRI, LRP could alleviate the ischemic brain edema and down-regulate the AQP4 expression, suggesting that LRP may inhibit the brain edema formation through down-regulating the AQP4 expression. This study demonstrated that LRP can delay or ameliorate the development of ischemic brain edema and it may be achieved by down-regulating the expression of AQP4. That is, the neuroprotective effects of LRP may result, in part, from its anti-AQP4 mechanisms. This study provides further evidence that LRP can be an effective therapy for stroke.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-36.
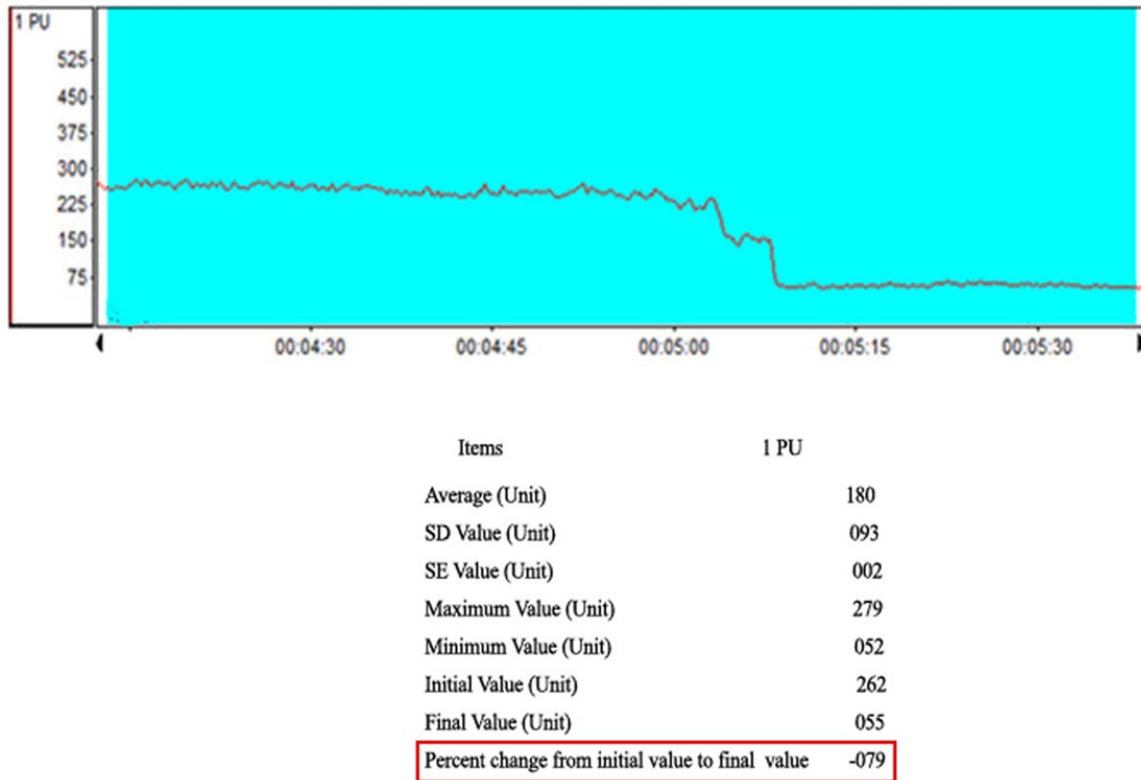
[2] Roth S, Li B, Rosenbaum PS, Gupta H, Goldstein IM, Maxwell KM, Gidday JM. Preconditioning provides complete protection against retinal ischemic injury in rats. Invest Ophthalmol Vis Sci 1998; 39: 777-85.

[3] Mounsey RA, Pang CY, Boyd JB, Forrest C. Augmentation of skeletal muscle survival in the latissimus dorsi porcine model using acute ischemic preconditioning. J Otolaryngol 1992; 21: 315-20.

[4] Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. Circ Res 1990; 66: 1133-42.

[5] Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. Circulation 1996; 94: 2193-200.

[6] Ates E, Genc E, Erkasap N, Erkasap S, Akman S, Firat P, Emre S, Kiper H. Renal protection by brief liver ischemia in rats. Transplantation 2002; 74: 1247-51.

[7] Brzozowski T, Konturek PC, Konturek SJ, Pajdo R, Kwiecien S, Pawlik M, Drozdowicz D, Sliwowski Z, Pawlik WW. Ischemic preconditioning of remote organs attenuates gastric ischemia-reperfusion injury through involvement of prostaglandins and sensory nerves. Eur J Pharmacol 2004; 499: 201-13.

[8] Waldow T, Alexiou K, Witt W, Albrecht S, Wagner F, Knaut M, Matschke K. Protection against acute porcine lung ischemia/reperfusion injury by systemic preconditioning via hind limb ischemia. Transpl Int 2005; 18: 198-205.

[9] Wei D, Ren C, Chen X, Zhao H. The chronic protective effects of limb remote preconditioning and the underlying mechanisms involved in inflammatory factors in rat stroke. PLoS One 2012; 7: e30892.

[10] Ren C, Gao X, Steinberg GK, Zhao H. Limb remote-preconditioning protects against focal ischemia in rats and contradicts the dogma of therapeutic time windows for preconditioning. Neuroscience 2008; 151: 1099-103.

[11] Malhotra S, Naggar I, Stewart M, Rosenbaum DM. Neurogenic pathway mediated remote preconditioning protects the brain from transient focal ischemic injury. Brain Res 2011; 1386: 184-90.

[12] Kakimoto M, Kawaguchi M, Sakamoto T, Inoue S, Furuya H, Nakamura M, Konishi N. Evaluation of rapid ischemic preconditioning in a rabbit model of spinal cord ischemia. Anesthesiology 2003; 99: 1112-7.

[13] Jin RL, Li WB, Li QJ, Zhang M, Xian XH, Sun XC, Zhao HG, Qi J. The role of extracellular signal-regulated kinases in the neuroprotection of limb ischemic preconditioning. Neurosci Res 2006; 55: 65-73.

[14] Dave KR, Saul I, Prado R, Busto R, Perez-Pinzon MA. Remote organ ischemic preconditioning protect brain from ischemic damage following asphyxial cardiac arrest. Neurosci Lett 2006; 404: 170-5.

[15] Vakili A, Mojarrad S, Akhavan MM, Rashidy-Pour A. Pentoxifylline attenuates TNF-alpha protein levels and brain edema following temporary focal cerebral ischemia in rats. Brain Res 2011; 1377: 119-25.

[16] Kahle KT, Simard JM, Staley KJ, Nahed BV, Jones PS, Sun D. Molecular mechanisms of ischemic cerebral edema: role of electroneutral ion transport. Physiology (Bethesda, Md) 2009; 24: 257-65.

[17] He Z, Wang X, Wu Y, Jia J, Hu Y, Yang X, Li J, Fan M, Zhang L, Guo J, Leung MC. Treadmill pretraining ameliorates brain edema in ischemic stroke via down-regulation of aquaporin-4: an MRI study in rats. PLoS One 2014; 9: e84602.

[18] Phillips MD, Zimmerman RA. Diffusion imaging in pediatric hypoxic ischemia injury. Neuroimaging Clin N Am 1999; 9: 41-52.

[19] Badaut J, Ashwal S, Tone B, Regli L, Tian HR, Obenaus A. Temporal and regional evolution of aquaporin-4 expression and magnetic resonance imaging in a rat pup model of neonatal stroke. Pediatr Res 2007; 62: 248-54.

[20] Papadopoulos MC, Verkman AS. Aquaporin-4 and brain edema. Pediatr Nephrol 2007; 22: 778-84.

[21] Meng S, Qiao M, Lin L, Del Bigio MR, Tomanek B, Tuor UI. Correspondence of AQP4 expression and hypoxic-ischaemic brain oedema monitored by magnetic resonance imaging in the immature and juvenile rat. Eur J Neurosci 2004; 19: 2261-9.

[22] Meller R, Simon RP. A critical review of mechanisms regulating remote preconditioning-induced brain protection. J Appl Physiol 2015; 119: 1135-42.

[23] Liu ZJ, Chen C, Li XR, Ran YY, Xu T, Zhang Y, Du HS, Leak RK, Ji XM, Hu XM. Remote Ischemic Preconditioning-Mediated Neuroprotection against Stroke is Associated with Significant Alterations in Peripheral Immune Responses. CNS Neurosci Ther 2016; 22: 43-52.

[24] Garcia-Bonilla L, Benakis C, Moore J, Iadecola C, Anrather J. Immune mechanisms in cerebral ischemic tolerance. Front Neurosci 2014; 8: 44.

[25] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20: 84-91.

[26] Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 1993; 87: 893-9.

[27] Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B. Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. Am J Physiol 1997; 273: H1707-12.

[28] Marmarou A. A review of progress in understanding the pathophysiology and treatment of brain edema. Neurosurg Focus 2007; 22: E1.

[29] Vella J, Zammit C, Di Giovanni G, Muscat R, Valentino M. The central role of aquaporins in the pathophysiology of ischemic stroke. Front Cell Neurosci 2015; 9: 108.

[30] Badaut J, Ashwal S, Obenaus A. Aquaporins in cerebrovascular disease: a target for treatment of brain edema? Cerebrovasc Dis (Basel, Switzerland) 2011; 31: 521-31.

[31] Obenaus A, Ashwal S. Magnetic resonance imaging in cerebral ischemia: focus on neonates. Neuropharmacology 2008; 55: 271-80.

[32] Zador Z, Stiver S, Wang V, Manley GT. Role of aquaporin-4 in cerebral edema and stroke. Handb Exp Pharmacol 2009; 159-70.

[33] Tait MJ, Saadoun S, Bell BA, Papadopoulos MC. Water movements in the brain: role of aquaporins. Trends Neurosci 2008; 31: 37-43.

[34] Hirt L, Ternon B, Price M, Mastour N, Brunet JF, Badaut J. Protective role of early aquaporin 4 induction against postischemic edema formation. J Cereb Blood Flow Metab 2009; 29: 423-33.

[35] Ribeiro Mde C, Hirt L, Bogousslavsky J, Regli L, Badaut J. Time course of aquaporin expression after transient focal cerebral ischemia in mice. J Neurosci Res 2006; 83: 1231-40.

[36] Friedman B, Schachtrup C, Tsai PS, Shih AY, Akassoglou K, Kleinfeld D, Lyden PD. Acute vascular disruption and aquaporin 4 loss after stroke. Stroke 2009; 40: 2182-90.

[37] Papadopoulos MC, Verkman AS. Aquaporin water channels in the nervous system. Nat Rev Neurosci 2013; 14: 265-77.

[38] Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P, Verkman AS. Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nat Med 2000; 6: 159-63.

[39] Nagelhus EA, Mathiisen TM, Ottersen OP. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. Neuroscience 2004; 129: 905-13.

[40] Igarashi H, Huber VJ, Tsujita M, Nakada T. Pretreatment with a novel aquaporin 4 inhibitor, TGN-020, significantly reduces ischemic cerebral edema. Neurol Sci 2011; 32: 113-6.

[41] Yao X, Derugin N, Manley GT, Verkman AS. Reduced brain edema and infarct volume in aquaporin-4 deficient mice after transient focal cerebral ischemia. Neurosci Lett 2015; 584: 368-72.

[42] Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999; 79: 1431-568.

[43] Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. Cardiovasc Res 2008; 79: 377-86.

[44] Wu W, Tian R, Hao S, Xu F, Mao X, Liu B. A pre-injury high ethanol intake in rats promotes brain edema following traumatic brain injury. Br J Neurosurg 2014; 28: 739-45.

[45] Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U, Meisel A. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 2002; 22: 10291-301.

[46] Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol 2000; 48: 285-96.

[47] Rite I, Machado A, Cano J, Venero JL. Intracerebral VEGF injection highly upregulates AQP4 mRNA and protein in the perivascular space and glia limitans externa. Neurochem Int 2008; 52: 897-903.

[48] Harrigan MR, Ennis SR, Sullivan SE, Keep RF. Effects of intraventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema, and infarct volume. Acta Neurochir 2003; 145: 49-53.

Items	1 PU
Average (Unit)	180
SD Value (Unit)	093
SE Value (Unit)	002
Maximum Value (Unit)	279
Minimum Value (Unit)	052
Initial Value (Unit)	262
Final Value (Unit)	055
Percent change from initial value to final value	-079

Figure S1. Brain perfusion was monitored by Laser Doppler Flowmeter. The picture upper showed that the cerebral blood perfusion was reduced to 79% after the MCA was occluded.