Effect of ischemic postconditioning on cerebral edema and the AQP4 expression following hypoxic-eschemic brain damage in neonatal rats

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Background: **A rat model for neonatal hypoxic-ischemic brain damage (HIBD) was established to observe the effect of ischemic postconditioning (IPostC) on cerebral edema and the AQP4 expression following HIBD and to verify the neuroprotection of IPostC and the relationship between changes of AQP4 expression and cerebral edema.**

Methods: **Water content was measured with dry-wet method, and AQP4 transcription and the protein expression of the lesions were detected with real-time PCR and immunohistochemistry staining, respectively.**

Results: **Within 6-48 hours, the degree of ipsilateral cerebral edema was significantly lower in IPostC-15 s/15 s group than in HIBD group. Similar to the HIBD group, the AQP4 transcription and expression in the IPostC group showed a downward and then upward trend. But the expression was still more evident in the HIBD group than in the IPostC-15 s/15 s group. From 24 to 48 hours, IPostC-15 s/15 s decreased the slowing down expression of AQP4 .**

Conclusion: **IPostC has neuroprotective effect on neonatal rats with HIBD and it may relieve cerebral edema by regulating the expression of AQP4.**

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Key words: **AQP4;**
cerebral edema;
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Introduction

Cerebral edema is a prominent feature during early pathological process in hypoxic-ischemic brain damage (HIBD), and early control of edema could improve the prognosis of the patient.[1] The main treatment options like dehydration and sub-hypothermia therapies have limited efficacy and thus novel therapeutics are required to control the molecular mechanisms underlying cerebral edema.[2] Evidence suggested that water channel protein aquaporins (AQPs) provided conduit for water transport through cytoplasm membrane. Among the AQPs family, AQP4 that is widely distributed across the brain tissues and mainly expressed on satellite cells in a polarized fashion plays a critical role in the central nervous system. It has been revealed that AQP4 involves in cerebral water movement but its effect is somewhat complicated. While it could promote the formation of cytotoxic edema, AQP4 is also associated with elimination of fluids when vasogenic edema occurs.[3] The further elucidation of AQP4 in cerebral edema might provide insights into the treatment of HIBD.

In essence, the pathogenesis of HIBD involves ischemia/reperfusion injury (IRI) and thereafter exacerbation of cerebral edema. Ischemic postconditioning (IPostC), an endogenous protective strategy, has been intensely studied in various diseases related to IRI. IPostC refers to alternative vascular occlusion and reperfusion after a period of inadequate blood flow in order to alleviate IRI, which is attracting much attention considering that it could be adopted after ischemia. IPostC has been shown to exert benefits after ischemia of the brain and spinal cord.[4-6] Liu et al and Xing et al[7,8] found that IPostC is able to ameliorate damage and decrease the area of infarction after focal ischemic injury in the brain, and other studies confirmed its neuroprotective role for global cerebral ischemia. The various mechanisms of IPostC preventing neuron loss include reducing apoptosis,[9] inhibiting brain inflammation[10] and modulating IRI-related molecules.[11]

AQP4 participates in brain IRI and the change of AQP4 distribution is closely associated with secondary cerebral edema.[12,13] Because of the limited antioxidant capacity in the developing brain of infants, the effect of IPostC on AQP4 expression and edema in HIBD is of particular importance.

Methods

Animal models and postconditioning protocol

Infant Sprague-Dawley rat pups weighing 18 to 22 g (7 days old) were obtained commercially from the Laboratory Animal Center of Xi'an Jiaotong University and housed with free access of water under specific pathogen-free conditions. This study was in accordance with University Institutional care and Use Committee on Animals. Altogether 480 rats were randomly divided into 4 groups: sham operation group (n=120), HIBD group (n=120), IPostC-15 s/15 s group (n=120) and IPostC-30 s/10 s group (n=120). Each group was further evenly divided into 5 subgroups based on the time interval between intervention and sacrifice, which is 0 hour, 6 hours, 12 hours, 24 hours and 48 hours, respectively (Fig. 1). HIBD was induced by the following procedure. Briefly, middle cervical incision was made in anesthesized rats and the right common carotid artery (CCA) was exposed. The ipislateral common carotid artery was ligated with a stainless vascular clamp. After occlusion of the right CCA, the animals were maintained in a hypoxia chamber with 8% oxygen and 92% nitrogen for 60 minutes. Then postconditioning was performed by repetitive brief release and occlusion (15 seconds or 30 seconds) of CCA. The procedure was repeated for 3 cycles. When rat pups were sacrificed, 0.9% NaCl was administered into left ventricle until the fluid in external jugular vein became transparent and then the fresh brain tissues were harvested for measurement of water content and quatitative Reverse Transcription-PCR (qRT-PCR).

Measurement of water content in brain tissues

Fresh brain specimens were weighted immediately after removal to obtain wet weight. Then, these specimens were dried in an oven at 120 °C for 24 hours and then reweighed to obtain the dry weight. Cerebral water content was calculated according to the following formula: [(wet weight-dry weight)]/wet weight×100%.

qRT-PCR

Total RNA from brain tissues was isolated and reverse transcribed using TRIzol reagents (Invitrogen) and PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China). The primers for AQP4 and β-actin were designed and purchased from Takara (Table). Real-time quantitative PCR analysis was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with melting curve analysis. The $-2^{-\Delta\Delta Ct}$ method was adopted to calculate the fold change in gene expression between the groups. AQP4 mRNA expression levels were normalized to that of β-actin. Each treatment was assayed in triplicate and three independent experiments were performed.

Immunohistochemistry

For immunohistochemical examinations, animals were transcardially perfused with 4% paraformaldehyde following perfusion with ice-cold normal saline solutions. Subsequently, brain samples were removed out, fixed and paraffin embedded. Each brain was cut into serial coronal sections at thickness of 4 μm. After deparaffinization with xylene and rehydration

Table. The sequence for aquaporin-4 (AQP4) and β-actin

Genes	Primers	Sequences (5' to 3')
AQP4	Forward primer	GCTGTGATTCCAAACGGACT
	Backward primer	CAGTGGTTTTCCCAGTTTCC
β-actin	Forward primer	CCCATCTATGAGGGTTACGC
	Backward primer	TTTAATGTCACGCACGATTTC

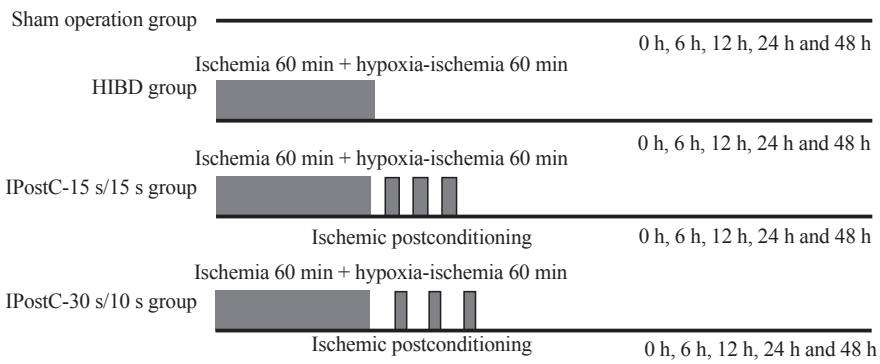

Fig. 1. Illustration of protocol for animal studies. HIBD: hypoxic-ischemic brain damage; IPostC: ischemic postconditioning.

with ethanol, the sections were incubated with a rabbit polycolonal AQP4 antibody (diluted 1:500; Millipore) overnight at 37°C. The sections were then incubated with horseradish peroxidase-conjugated antibodies against rabbit immunoglobulin using Histostain-Plus Kit (ZSGB-BIO, Beijing, China). Finally, the sections were counterstained with hematoxylin. The micrographs were captured by a digital microscope system (Leica Microsystems, Wetzlar, Germany) in the region of parietal cortex. Image-Pro Plus 6.0 software (Media Cybernetics, USA) was used to semi-quantitatively evaluate AQP4 expression. Specifically, optical density (OD) at a high power field in stained sections was calculated and the sum of OD that is associated with AQP4 expression was synthesized.

Statistical analysis

All data were presented as mean with standard deviation. Statistical calculations were performed using SPSS 13.0 (SPSS Inc.). Independent 2-sample *t* test compared differences between two groups, and one-way ANOVA with the least significant difference test for *post hoc* comparisons compared differences between multiple groups. For all tests, a *P* value below 0.05 was considered statistically significant.

Results

Brain morphological changes

In each time point after reperfusion, the rats in the sham operation group exhibited normal cerebral morphology, whereas those in the HIBD group showed obvious right cerebral aberrations including swelling and malacia lesions. While the IPostC-30 s/10 s group presented similar damage of the right brain as the HIBD group, rats undergoing IPostC-15 s/15 s had markedly attenuated injury compared with those in the HIBD group.

IPostC-15s/15s decreases brain water content

The animals in the sham operation group had a comparable level of brain water content before and after reperfusion ($P>0.05$) (Fig. 2A). By contrast, for other three groups, there were increases in water content following reperfusion with similar fashion. Water content rises sharply from 0 to 6 hours and decreases in 12 hours, and then increases again to 48 hours maintained at the highest level. The brain water contents are similar at 0 hour after reperfusion except for the sham operation group, but from 6 hours, the level in the IPostC-15 s/15 s group was consistently lower than that in the HIBD and IPostC-30 s/10 s groups ($P<0.05$). In the comparison between the HIBD and IPostC-30 s/10 s groups, no significant difference was observed ($P>0.05$).

AQP4 mRNA expression in each group

Real-time PCR was performed to analyze AQP4 mRNA expression in different groups (Fig. 2B). There were no marked changes at each time point in the sham operation group, and the post-reperfusion expression level was consistently lower than that in other groups ($P<0.01$). In other three groups, however, similar trends were observed toward increased AQP4 expression cumulating at 6 hours after reperfusion followed by a reduction at 12 hours and a secondary increase from then onward. The peak value was significantly higher in the HIBD group than in other two groups with treatment of postconditioning. Subsequently, a significant reduction in expression level at 12 hours was found in the HIBD and IPostC-30 s/10 s groups ($P<0.01$) but not in the IPostC-15 s/15 s group. At 12 and 24 hours, AQP4 mRNA expression was significantly higher in the HIBD group than in the IPostC-15 s/15 s group ($P<0.01$), but it was only slightly upregulated compared with the IPostC-30 s/10 s group ($P>0.05$). After 24 hours, the three groups showed decreased expression with a prominent reduction in the IPostC-15 s/15 s group though the

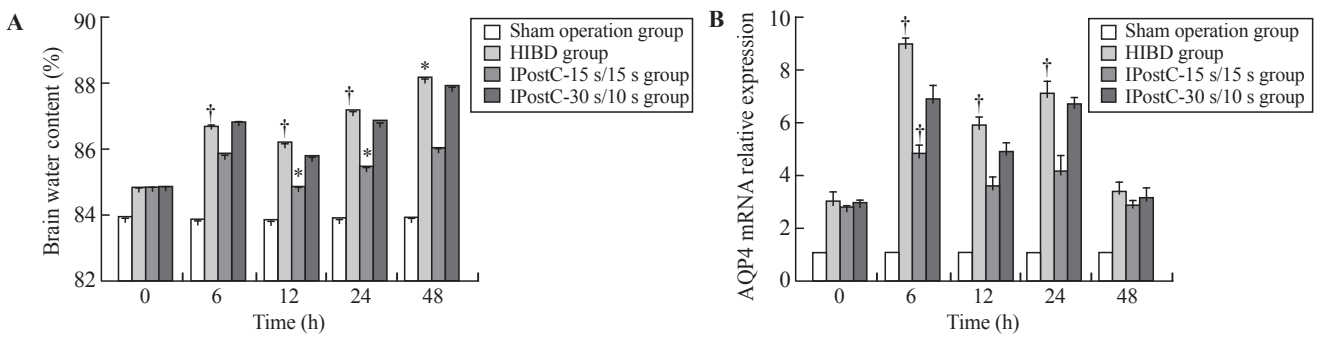

Fig. 2. A: Brain water content of rats with different treatments; **B:** AQP4 mRNA expression in brain tissues of rats with different treatments. *: $P<0.05$; †: $P<0.01$. AQP4: aquaporin-4; HIBD: hypoxic-ischemic brain damage; IPostC: ischemic postconditioning.

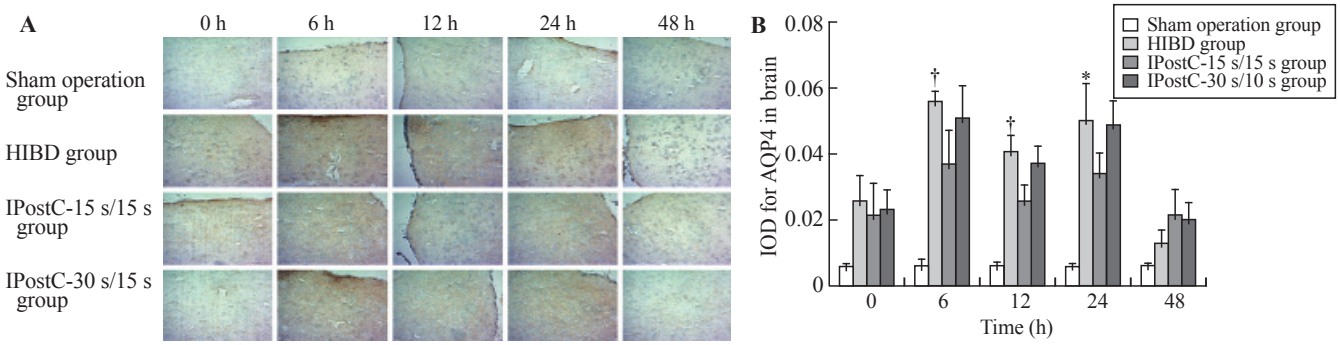

Fig. 3. A: Immunohistochemistry of AQP for brain specimens of animals with different treatments; **B:** Comparison of AQP expression in immunohisochemical examinations. *: $P<0.05$; †: $P<0.01$. AQP4: aquaporin-4; HIBD: hypoxic-ischemic brain damage; IPostC: ischemic postconditioning; IOD: integrated optical density.

differences were not significant in comparison to other two groups.

AQP4 protein expression in each group

Inmmunohistochemistry was applied to evaluate AQP4 protein expression in different groups (Fig. 3A and B). The results demonstrated that AOP4 was expressed on gilal cells and the perivascular glial cell plate. There was only scatted positive immunostaining in the sham operation group, which was remarkably lower than that of other three groups at almost all post-reperfusion time points ($P<0.05$). The protein expression increased after reperfusion with a temporary reduction at 12 hours which was similar as mRNA expression pattern. Besides 0 and 48 hours, immunostaining was comparable ($P>0.05$) in the HIBD and IPostC-30 s/10 s groups and was markedly stronger than that in the IPostC-15 s/15 s group ($P<0.05$). The three groups exhibited attenuated AQP4 immunopositivity at 48 hours, and among them, the most prominent decrease presented in the HIBD group. Only slight reduction occurred in the IPostC-15 s/15 s group, and the difference compared with 24 hours was not significant ($P>0.05$).

Discussion

Cerebral edema in HIBD is a mixed pattern with simultaneous cytotoxic and vasogenic edema. While cytotoxic edema is in dominance with swelling of endothelial cells and glia cells as well as neurons during the early period of HIBD, vasogenic edema is predominant lately along with blood-brain barrier damage and vascular hyperpermeability.[14] Given the dual regulatory effects of AQP4 on water homeostasis in the central nervous system, we speculate that up-regulated AQP4 contributes to the formation of cytotoxic edema and elimination of fluids in early and late stage of HIBD, respectively.

In the current study, we established a neonatal rat HIBD model via inducing cerebral ischemia-reperfusion injury. AQP4 expression was substantially up-regulated 6 hours after reperfusion with simultaneous increase of brain water content, which accounts for the development of cytotoxic edema within 24 hours after hypoxic-ischemic injury. AQP4 expression was down-regulated between 6 to 12 hours, which might be explained by a self-protection mechanism to restrict cellular swelling and inhibit progression of cerebral edema. However, the protection was limited and thus brain water content was elevated again from 12 to 24 hours. Although the level of AQP expression markedly reduced at 48 hours, brain edema became aggravated without attenuation. It is generally believed that 24 hours after reperfusion secondary energy metabolism failure occurs and Na^+, K^+-ATP enzyme activity diminishes, both of which contribute to the reduction of AQP4 synthesis. The decreased level of AQP4 expression delays the elimination of fluids from brain parenchyma. At the same time, accumulation of free radical, excitatory neurotoxicity and inflammatory reactions exacerbate cerebral edema. In addition, up-regulation of hypoxia-induced factor 1-α induced by HIBD is associated with down-regulated AQP4.[15]

To validate the hypotheses that IPostC could alleviate brain damage via reducing edema after ischemia, Ren et al[16] found that IPostC enhanced glucose intake and depressed edema development and BBB destruction. It has been demonstrated that rats undergoing IPostC showed significant improvement of cerebral edema in a model of IRI.[17] The present study implemented two different postconditioning methods, IPostC-15 s/15 s and IPostC-30 s/10 s, both of which had similar trends in change of brain water content as the HIBD group without any interventions. Compared with the HIBD group, the IPostC-15 s/15 s group displayed a remission of edema but the IPostC-30 s/10 s group did not. Except for the time points of 0 and 48 hours, AQP4 expression was significantly decreased in

the IPostC-15 s/15 s group compared with the HIBD group whereas it was not decreased obviously in the IPostC-30 s/10 s group. Nevertheless, animals in the IPostC-15 s/15 s group had a comparable AQP4 level when vasogenic edema was predominant. Therefore, IPostC-15 s/15 s but not IPostC-30 s/10 s appears to be benefitial to ischemic injury. The underlying mechanisms for outcome discrepancy between the two intervention groups were not investigated in the present study. Presumably, prolonged ischemia in the IPostC-30 s/10 s group could contribute to the ineffectiveness of post-conditioning, though the ischemic time was increased only by 15 seconds. In fact, other research groups have demonstrated that elevated ischemic time abrogated the benefits of post-conditioning in a time-dependent manner in brain IRI including inhibition of neuronal apoptosis.[9]

Our results confirmed that IPostC could prevent HIBD through AQP4 regulation to improve cerebral edema. But the mechanism underlying this protective effect remains to be defined. It has been revealed that the neuroprotective effect of IPostC is associated with activation of PKC that triggers the phosphorylation of AQP4 and its degradation by lysosome.[18] Also, IPostC is able to attenuate post-ischemic injury via down-regulating co-transporters of Na^+, K^+, Cl^+ (NKCC1).[19] Because NKCC1 on astrocytes coordinate with AQP4 to mediate water transport, IPostC might regulate AQP4 by decreasing the expression of NKCC1.[20] Finally, the PI3K/Akt pathway was implicated in mediating neuroprotective effects of postconditioning and its activation could contribute to AQP4 down-regulation by increasing the HIF-1α level.[15]

The mechanism for slow decrease of AQP4 expression in a late period of reperfusion is not completely understood. When cerebral ischemia presents, matrix metalloproteinase-9 (MMP-9) participates in degradation of fibronectin and type IV collagen on microvascular basal membrane, thereby resulting in impairment of blood-brain barrier (BBB) and vasogenic edema. It has been shown that IPostC alleviated cerebral edema through down-regulation of MMP-9. Additionally, astrocytes are believed to exert a significant role in inducing and maintaining integrity of BBB. As a result, IPostC might modulate the auto-regulation of astrocytes on AQP4 via MMP-9 down-regulation.

In conclusion, IPostC has neuroprotective effect on neonatal rats with HIBD and it may relieve cerebral edema by regulating the expression of AQP4.

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Ethical approval: The animal studies were in accordance with the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Contributors: LL and LY proposed the study. YL and FSJ performed the animal studies and other research. YL, XM, LXJ, CXL and LHX collected and analyzed the data. YL and LJX wrote the first draft. LL made the revision and final proofreading of the article. LL is the guarantor.

References

1 Fu X, Li Q, Feng Z, Mu D. The roles of aquaporin-4 in brain edema following neonatal hypoxia ischemia and reoxygenation in a cultured rat astrocyte model. Glia 2007;55:935-941.
2 Nijboer CH, van der Kooij MA, van Bel F, Ohl F, Heijnen CJ, Kavelaars A. Inhibition of the JNK/AP-1 pathway reduces neuronal death and improves behavioral outcome after neonatal hypoxic-ischemic brain injury. Brain Behav Immun 2010;24:812-821.
3 Saadoun S, Papadopoulos MC. Aquaporin-4 in brain and spinal cord oedema. Neuroscience 2010;168:1036-1046.
4 Zhao H, Sapolsky RM, Steinberg GK. Interrupting reperfusion as a stroke therapy: ischemic postconditioning reduces infarct size after focal ischemia in rats. J Cereb Blood Flow Metab 2006;26:1114-1121.
5 Jiang X, Shi E, Nakajima Y, Sato S. Postconditioning, a series of brief interruptions of early reperfusion, prevents neurologic injury after spinal cord ischemia. Ann Surg 2006;244:148-153.
6 Yang F, Zhang X, Sun Y, Wang B, Zhou C, Luo Y, et al. Ischemic postconditioning decreases cerebral edema and brain blood barrier disruption caused by relief of carotid stenosis in a rat model of cerebral hypoperfusion. PLoS One 2013;8:e57869.
7 Liu XR, Luo M, Yan F, Zhang CC, Li SJ, Zhao HP, et al. Ischemic postconditioning diminishes matrix metalloproteinase 9 expression and attenuates loss of the extracellular matrix proteins in rats following middle cerebral artery occlusion and reperfusion. CNS Neurosci Ther 2012;18:855-863.
8 Xing B, Chen H, Zhang M, Zhao D, Jiang R, Liu X, et al. Ischemic postconditioning inhibits apoptosis after focal cerebral ischemia/reperfusion injury in the rat. Stroke 2008;39:2362-2369.
9 Wang JY, Shen J, Gao Q, Ye ZG, Yang SY, Liang HW, et al. Ischemic postconditioning protects against global cerebral ischemia/reperfusion-induced injury in rats. Stroke 2008;39:983-990.
10 Joo SP, Xie W, Xiong X, Xu B, Zhao H. Ischemic postconditioning protects against focal cerebral ischemia by inhibiting brain inflammation while attenuating peripheral lymphopenia in mice. Neuroscience 2013;243:149-157.
11 Kong Y, Rogers MR, Qin X. Effective neuroprotection by ischemic postconditioning is associated with a decreased expression of RGMa and inflammation mediators in ischemic rats. Neurochem Res 2013;38:815-825.
12 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;83:1231-1240.
13 Frydenlund DS, Bhardwaj A, Otsuka T, Mylonakou MN, Yasumura T, Davidson KG, et al. Temporary loss of perivascular

aquaporin-4 in neocortex after transient middle cerebral artery occlusion in mice. Proc Natl Acad Sci U S A 2006;103:13532-13536.
14 Papadopoulos MC, Verkman AS. Aquaporin-4 and brain edema. Pediatr Nephrol 2007;22:778-784.
15 Gao X, Zhang H, Takahashi T, Hsieh J, Liao J, Steinberg GK, et al. The Akt signaling pathway contributes to postconditioning's protection against stroke; the protection is associated with the MAPK and PKC pathways. J Neurochem 2008;105:943-955.
16 Ren C, Gao X, Niu G, Yan Z, Chen X, Zhao H. Delayed postconditioning protects against focal ischemic brain injury in rats. PLoS One 2008;3:e3851.
17 Tan X, Zhang L, Jiang Y, Yang Y, Zhang W, Li Y, et al. Postconditioning ameliorates mitochondrial DNA damage and deletion after renal ischemic injury. Nephrol Dial Transplant 2013;28:2754-2765.
18 Kaur S, Jaggi AS, Singh N. Molecular aspects of ischaemic postconditioning. Fundam Clin Pharmacol 2009;23:521-536.
19 Pignataro G, Scorziello A, Di Renzo G, Annunziato L. Post-ischemic brain damage: effect of ischemic preconditioning and postconditioning and identification of potential candidates for stroke therapy. FEBS J 2009;276:46-57.
20 Zelenina M. Regulation of brain aquaporins. Neurochem Int 2010;57:468-488.

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