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Gene interference regulates aquaporin-4 expression in swollen tissue of rats with cerebral ischemic edema

Correlation with variation in apparent diffusion coefficient☆

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Abstract

To investigate the effects of mRNA interference on aquaporin-4 expression in swollen tissue of rats with ischemic cerebral edema, and diagnose the significance of diffusion-weighted MRI, we injected 5 μL shRNA- aquaporin-4 (control group) or siRNA- aquaporin-4 solution (1:800) (RNA interference group) into the rat right basal ganglia immediately before occlusion of the middle cerebral artery. At 0.25 hours after occlusion of the middle cerebral artery, diffusion-weighted MRI displayed a high signal; within 2 hours, the relative apparent diffusion coefficient decreased markedly, aquaporin-4 expression increased rapidly, and intracellular edema was obviously aggravated; at 4 and 6 hours, the relative apparent diffusion coefficient slowly returned to control levels, aquaporin-4 expression slightly increased, and angioedema was observed. In the RNA interference group, during 0.25–6 hours after injection of siRNA- aquaporin-4 solution, the relative apparent diffusion coefficient slightly fluctuated and aquaporin-4 expression was upregulated; during 0.5–4 hours, the relative apparent diffusion coefficient was significantly higher, while aquaporin-4 expression was significantly lower when compared with the control group, and intracellular edema was markedly reduced; at 0.25 and 6 hours, the relative apparent diffusion coefficient and aquaporin-4 expression were similar when compared with the control group; obvious angioedema remained at 6 hours. Pearson's correlation test results showed that aquaporin-4 expression was negatively correlated with the apparent diffusion coefficient ($r = -0.806$, $P < 0.01$). These findings suggest that upregulated aquaporin-4 expression is likely to be the main molecular mechanism of intracellular edema and may be the molecular basis for decreased relative apparent diffusion coefficient. Aquaporin-4 gene interference can effectively inhibit the upregulation of aquaporin-4 expression during the stage of intracellular edema with time-effectiveness. Moreover, diffusion-weighted MRI can accurately detect intracellular edema.

Key Words

cerebral ischemic edema; magnetic resonance imaging; diffusion; gene silencing; aquaporin-4; mRNA interference; neural regeneration

Research Highlights

(1) Upregulation of aquaporin-4 expression is the main molecular mechanism underlying intracellular edema, and is also the molecular basis for decreased relative apparent diffusion coefficient. (2) Aquaporin-4 gene interference can effectively inhibit the upregulation of aquaporin-4 expression during the stage of intracellular edema in cerebral ischemia. (3) Diffusion-weighted MRI can accurately monitor intracellular edema.

Abbreviations

DW-MRI, diffusion-weighted MRI; MCAO: middle cerebral artery occlusion

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INTRODUCTION

Brain edema, a common pathological phenomenon in cerebral ischemia, can result in serious outcomes[1-2]. Glial cell edema is the earliest manifestation in ischemic cerebrovascular disease, which is related to aquaporin-4. Diffusion-weighted MRI (DW-MRI) can detect the degree of glial cell edema, and it has been shown that the apparent diffusion coefficient is negatively related with aquaporin-4 expression[3-10]. This finding may provide a theoretical basis for early diagnosis of cerebral infarction, accurate determination of a “therapeutic window”, and prompt rescue of ischemic brain tissue. However, the key molecular mechanism of aquaporin-4 during the early stages of ischemic cerebral edema remains unclear. Therefore, we used RNA interference to dynamically detect apparent diffusion coefficient changes and aquaporin-4 expression in ischemic rat brain tissue through the use of DW-MRI to evaluate the function of the aquaporin-4 gene.

RESULTS

Quantitative analysis of experimental animals

A total of 108 healthy Wistar rats were randomly divided into three groups: ischemia group (MCAO), control group (shRNA-aquaporin-4 + MCAO), and RNA interference group (siRNA-aquaporin-4 + MCAO). Cerebral ischemia was induced in all rats by occlusion of the right cerebral middle artery. In the latter two groups, prior to MCAO, shRNA-containing liposome solution and siRNA-aquaporin-4-containing liposome solution were injected into the right basal ganglia in the control and RNA interference groups, respectively. Each group was divided into six subgroups as per time points (0.25, 0.5, 1, 2, 4 and 6 hours after MCAO), with six rats in each subgroup. Rats lost due to failed cerebral ischemia induction were supplemented in time, and altogether 108 rats were included in the final analysis.

DW-MRI findings and relative apparent diffusion coefficient value alteration in rats with cerebral ischemic edema

There was no significant difference in relative apparent diffusion coefficient values between the control and ischemia groups ($P > 0.05$). DW-MRI showed a high signal at 0.25 hours after MCAO and the signal increased gradually as time progressed. In the RNA interference group, the area with a high signal on DW-MRI increased slightly but not significantly between 0.25–2 hours and 4–6 hours. relative apparent diffusion coefficient values increased in the RNA interference group compared with the control group between 0.5 hours and 4 hours ($P < 0.01$) (Figures 1, 2).

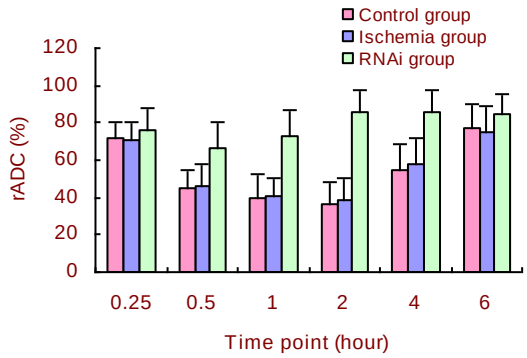

Figure 1 Relative apparent diffusion coefficient value (rADC) in each group at different time points.

Data are expressed as mean ± SD of six rats at each time point. Repeated-measures analysis of variance was used.

The change in rADC values was consistent between the control and ischemia groups ($P > 0.05$), however, rADC values rapidly declined during 0.25–2 hours, and there was a significant difference at 0.5 hours and 4 hours between the control and RNA interference (RNAi) groups ($P < 0.01$).

rADC values rebounded during 4–6 hours, and there was no significant difference at 0.25 and 6 hours between the control and RNAi groups ($P > 0.05$).

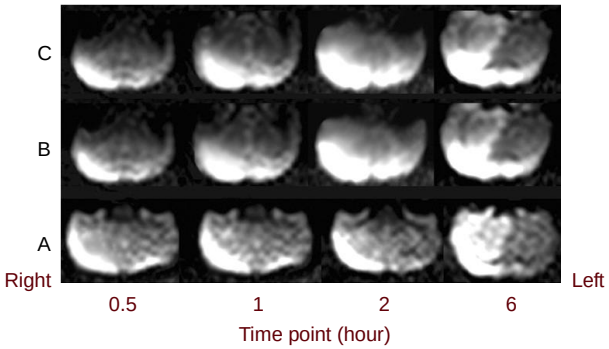

Figure 2 Diffusion-weighted (DW) MRI in RNA interference (A), ischemia (B) and control (C) groups.

Hyper-intense signal ranges of DW-MRI were consistent between the ischemia and control groups.

The control group exhibited a high signal in the right basal ganglia at 0.5 hours after middle cerebral artery occlusion. The scope and intensity of the high signal area increased rapidly within 2 hours, while the parameter of RNA interference group showed no increasing rapidly during this period.

Pathological findings in cerebral ischemic edema tissue

Pathological changes in the ischemia group were the same as the control group. At 0.25 hours after MCAO in the ischemia group, a small amount of swollen glial cells with a round appearance, enlarged soma, and a light eosinophilic

cytoplasm were observed under the optical microscope (Figure 3). At 0.25 hours after MCAO, mitochondrial swelling, vacuole-like changes, expanded endoplasmic reticulum, nuclear swelling and chromatin margination were observed under the electron microscope (Figure 4).

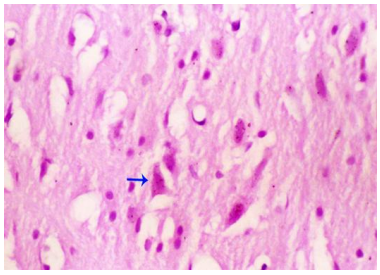

Figure 3 Astrocytic swelling (arrow) and a widened pericellular interspace indicate intracellular edema in the ischemia group at 0.25 hours after middle cerebral artery occlusion (hematoxylin-eosin staining, × 400).

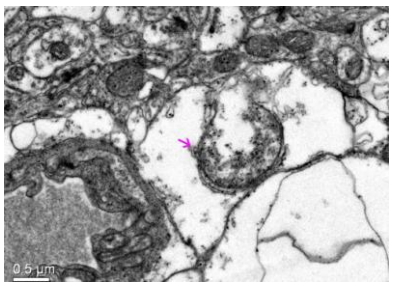

Figure 4 Mitochondrial swelling and vacuoles (arrow) indicate intracellular edema in the ischemia group at 0.25 hours after middle cerebral artery occlusion (transmission electron microscopy, × 6 000).

At 1 hour, dark stained nuclei, and eosinophilic changes in parts of the cytoplasm (red neurons) were observed under the optical microscope. In addition, organelle swelling of entire membranes was observed under the electron microscope. At 2 hours, nuclear condensation and glial cell swelling became more evident, light transmission appeared around the cells, and intercellular space decreased; however, the cell membrane remained intact. At 4 hours, endothelial cells appeared swollen, the space between blood vessels and cells expanded, glial cell membranes became swollen and thick, vessels were compressed and deformed, and a light red mesh-like structure emerged in the tissue space, which indicated angioedema. Under the electron microscope, partly ruptured cell membranes, karyolysis, chromatin margination, appearance of medullary structures within the cytoplasm, and damage to the blood-brain barrier was observed (Figure 5). At 6 hours, the above changes (angioedema) continued to progress. Glial cell swelling in the RNA interference group was significantly alleviated compared with the control group at each time point, in particular within 2 hours (Figure 6).

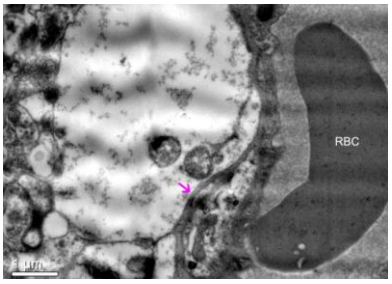

Figure 5 Vascular endothelial swelling and blood-brain barrier damage (arrow) indicate angioedema in the ischemia group at 4 hours after middle cerebral artery occlusion (transmission electron microscope, × 10 000). RBC: Red blood cell.

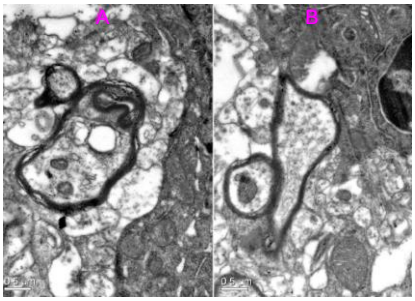

Figure 6 Comparison between control group (A) and RNA interference group (B) at 2 hours after middle cerebral artery occlusion:

In the control group, mitochondrial swelling, vacuole-like changes, and chromatin margination are observed.

In the RNA interference group, mitochondrial swelling and vacuole-like changes (glial cell edema) significantly reduced when compared with the control group (transmission electron microscope, × 6 000).

Aquaporin-4 mRNA expression in cerebral ischemic edema tissue

Real-time PCR showed that there was no significant difference in aquaporin-4 mRNA expression between the control and ischemia groups ($P > 0.05$). In the control group, aquaporin-4 mRNA expression increased quickly within 2 hours and slowly during 4–6 hours. In the RNA interference group, aquaporin-4 mRNA expression was significantly up regulated during 0.25–4 hours. Aquaporin-4 mRNA expression significantly decreased in the RNA interference group compared with the control group during 0.5–4 hours ($P < 0.05$; Figure 7).

Aquaporin-4 protein expression in cerebral ischemic edema tissue

Western blot analysis results showed that aquaporin-4 protein expression was consistent with gene expression, and there was no significant difference in aquaporin-4 protein expression between the control and ischemia groups ($P > 0.05$; Figure 8).

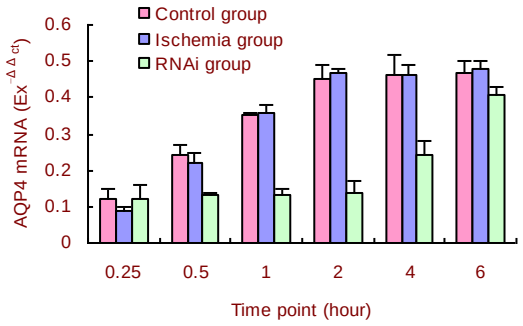

Figure 7 Aquaporin-4 (AQP4) mRNA expression in rat right basal ganglia tissue (infarct site).

Data are expressed as mean ± SD of six rats at each time point. Repeated-measures analysis of variance was used.

There was no significant difference in AQP4 mRNA expression between the control group and ischemia group ($P > 0.05$), as well as between the control group and RNA interference (RNAi) group at 0.25 hours and 6 hours ($P > 0.05$).

AQP4 mRNA expression was significantly higher in the control group than in the RNA interference group during 0.5–4 hours ($P < 0.05$).

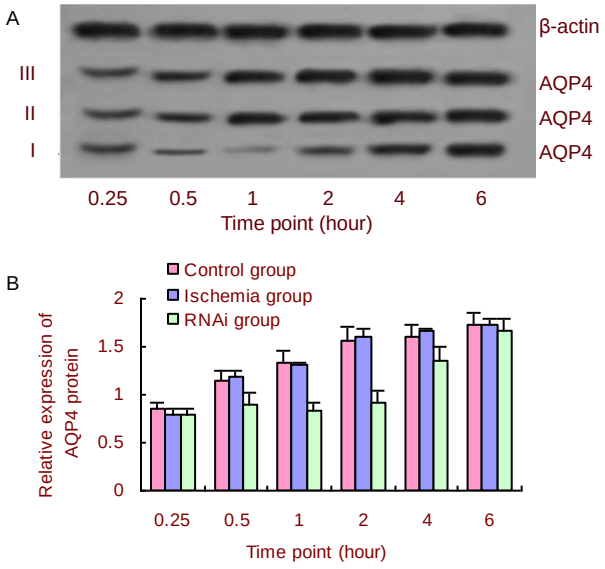

Figure 8 Aquaporin-4 (AQP4) protein expression in rat right basal ganglia tissue (infarct site).

(A) Western blot results of AQP4 protein expression in the RNA interference (RNAi, I), ischemia (II), and control (III) groups.

(B) Quantification of AQP4 protein expression.

Data are expressed as mean ±SD of six rats at each time point. Repeated-measures analysis of variance was used. Relative expression levels of proteins were expressed as the absorbance ratio of target protein to β-actin.

There was no significant difference in AQP4 protein expression between the control group and ischemia group ($P > 0.05$), as well as between the control group and RNAi group at 0.25 and 6 hours ($P > 0.05$).

AQP4 protein expression was significantly higher in the control group than in the RNAi group during 0.5–4 hours ($P < 0.01$).

In the control group, aquaporin-4 protein expression increased rapidly in almost a linear manner from 0.25 to 2 hours and increased slowly after 4 hours. In the RNA interference group, aquaporin-4 protein expression increased slowly at 0.25 and 6 hours. Aquaporin-4 protein expression was significantly higher in the control group than in the RNA interference group during 0.5–4 hours ($P < 0.01$) except at 0.25 hours and 6 hours ($P > 0.05$; Figure 8).

Correlation analysis between aquaporin-4 protein and relative apparent diffusion coefficient values

The regression equation showed a negative correlation between aquaporin-4 protein expression and the relative apparent diffusion coefficient value: absorbance value = – 68.90 relative apparent diffusion coefficient + 57.855 (Pearson's correlation test; $r = -0.806$, $P < 0.01$).

DISCUSSION

Pathological basis for diagnosis of ischemic brain edema using DW-MRI

The movement of diffused water molecules is often detected by apparent diffusion coefficient. Relative apparent diffusion coefficient shares the same meaning as apparent diffusion coefficient, but is more comprehensive and stable because it excludes local interference on the infarct side. To date, DW-MRI has become the most sensitive method to measure water apparent diffusion coefficient values of living tissue[11-12]. If water molecules can move or diffuse freely within the voxel and the phase is not lost, apparent diffusion coefficient values are large and DW-MRI signals will decrease. Conversely, if water molecule diffusion is limited, apparent diffusion coefficient values are smaller and DW-MRI signals will be increased. Theoretically, there is a negative exponential relationship between the signal intensity of DW-MRI and apparent diffusion coefficient values[13]. It has been reported that DW-MRI can detect the lesion as early as 2.7 minutes after ischemia, which almost synchronizes with the occurrence of intracellular edema[14-15]. Our previous study showed that intracellular edema is an early pathological change following cerebral ischemia, which may be the pathological basis for decreased apparent diffusion coefficient values and high DW-MRI signals[5]. Results from this study showed that at 2 hours after embolization, intracellular edema was a major pathological change in brain tissue, that relative apparent diffusion coefficient values of the infarcted area decreased rapidly with the degree of intracellular edema, and that high DW-MRI signal intensity increased. At 4 hours, relative apparent diffusion coefficient values

increased again, and angioedema occurred. aquaporin-4 siRNA significantly alleviated cell edema in the infracted region during 0.25–2 hours, and relative apparent diffusion coefficient values in the infarct region did not decrease, while DW-MRI signal range did not increase significantly. Intracellular edema is the main reason why apparent diffusion coefficient values decrease during early phases of cerebral ischemia, and the pathological basis of DW-MRI facilitates early diagnosis of cerebral infarction.

Effect of aquaporin-4 mRNA interference on aquaporin-4 expression in cerebral ischemic edema tissue and pathological changes

Pathophysiological studies have shown that after a few minutes of cerebral ischemia, Na^+-K^+-ATPase activity decreases, intracellular Na^+ remains the same, and a large quantity of free water molecules enter the cells, leading to intracellular edema[16]. However, the mechanism underlying intracellular edema is very complex and it is generally considered that water molecules infiltrate cells *via* simple diffusion or against an ionic concentration gradient. However, the osmotic water permeability coefficient of the biofilter is much greater than its diffusion permeability coefficient. Moreover, water diffusion requires energy (ATP), which cannot explain the instantaneous transport of transmembrane water that leads to intracellular edema, which challenges the traditional view. Many scholars have shown that aquaporin-4 is closely linked to the development of brain edema[4, 17]. In our previous study, using an ischemic-reperfusion mouse model, we found that aquaporin-4 expression in ischemic brain tissue is closely related to the degree of glial cell edema[18]. However, whether aquaporin-4 expression is the only key factor that causes ischemic brain edema remains uncertain. This study used gene interference to inhibit aquaporin-4 expression. Our results showed that aquaporin-4 gene and protein expression in the MCAO group rapidly increased within 2 hours, and that intracellular edema was obvious; while aquaporin-4 gene and protein expression in the RNA interference group increased slowly over the same time period. There was a significant difference in aquaporin-4 expression between the RNA interference group and control group during the period spanning 30 minutes to 4 hours (*i.e*. intracellular edema was the major change), and intracellular edema in the RNA interference group also significantly reduced. However, during 4–6 hours, aquaporin-4 expression in the RNA interference group showed a decreasing trend and began to induce the pathological manifestation of angioedema. These results show that aquaporin-4 gene interference can significantly inhibit aquaporin-4 protein expression during the intracellular edema stage and effectively alleviate intracellular edema, while alleviating inhibition of aquaporin-4 expression, during the angioedema phase. It is speculated that the pathological molecular mechanism during this period is not due to the single factor of aquaporin-4 expression. There may be participation of other factors (*e.g*. aquaporin-9, Ca^{2+}), which require further investigation. No significant differences in relative apparent diffusion coefficient at 15 minutes and aquaporin-4 expression were observed between the RNA interference group and control group. This may be because aquaporin-4 expression in brain tissue during the early infarction period is only just beginning, causing mild intracellular edema, and a decline in relative apparent diffusion coefficient values and coupled with aquaporin-4 gene interference, results in delayed interference on aquaporin-4 expression (in this experiment 0.5 hours to 4 hours to interfere significantly), which shows that aquaporin-4 gene interference is time-dependent.

Relationships among aquaporin-4, intracellular edema and apparent diffusion coefficient value

Previous studies have only focused on changes in apparent diffusion coefficient values or aquaporin-4 expression during early cerebral ischemia[19-21], however, most scholars believe that aquaporin-4 plays an important role in rapid transmembrane water transport in astrocytes[22-26]. Li *et al* [27] reported that aquaporin-4 expression and osmotic pressure were related. To date, there has been no report describing the relationship between pathological changes during the early cerebral ischemia (stage of intracellular edema), aquaporin-4 expression and apparent diffusion coefficient values. Results from this study showed that aquaporin-4 expression negatively correlated with relative apparent diffusion coefficient values significantly ($r = -0.806$, $P < 0.01$). We hypothesized that cerebral ischemia induces a decrease in Na^+-K^+-ATPase activity, which leads to changes in intracellular and extracellular ionic osmotic pressure, triggering upregulation of aquaporin-4 expression, resulting in intracellular edema and restricted diffusion of water molecules inside cells. As a result, apparent diffusion coefficient values decreased and high DW-MRI signals were observed. aquaporin-4 may be “the last common channel” for intracellular edema, and it is an important molecular mechanism that underlies the formation of intracellular edema. In addition, aquaporin-4 leads to decreased apparent diffusion coefficient values and increased DW-MRI signals (supplementary Figure 1 online). Aquaporin-4 gene interference can effectively inhibit aquaporin-4 expression during early stages of cerebral ischemia, which reduces intracellular edema, elevates apparent diffusion coefficient values in cerebral infarction tissue, and decreased DW-MRI signals.

Taken together, this study showed that high expression of aquaporin-4 is closely related to cerebral ischemic edema, and maybe a molecular basis for decreased relative apparent diffusion coefficient. Aquaporin-4 RNA interference could effectively inhibit the upregulation of aquaporin-4 expression. However, the inhibitory effect of RNA interference decreased when aquaporin-4 expression appeared in the angioedema during the late stage of cerebral ischemia. These findings provide evidence for the efficacy of gene therapy during early cerebral ischemia and the application of DW-MRI to monitor the extent of intracellular edema.

MATERIALS AND METHODS

Design

A controlled, repeated-measures, randomized animal study.

Time and setting

Experiments were performed from September 2009 to December 2011 in the Huaxi Medical Center, Sichuan University, and Affiliated Haikou Hospital of Xiangya School of Medicine, Central South University, China.

Materials

A total of 108 healthy Wistar rats, weighing 300–350 g, males and females, were purchased from the Animal Laboratory, West China Medical Center, Sichuan University, China (license No. SCXK (chuan) 2008-24). Rats were housed in clean cages at 23 ± 1°C and under a 12-hour light-dark cycle. All rats were fasted for 24 hours before surgery, and experimental protocols were performed in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[28].

Methods

Establishment of siRNA-aquaporin-4-MCAO animal model and RNA interference treatment

Rats were divided into an ischemia group (MCAO), control group (shRNA-aquaporin-4 + MCAO, shRNA-aquaporin-4 sequence:
5'-UUC UCC GAA CGU GUC ACG UTT-3'
5'-ACG UGA CAC GUU CGG AGA ATT-3')
and interference group (siRNA-aquaporin-4 + MCAO, siRNA-aquaporin-4 sequence:
5'-GAC AUU UGU UUG CAA UCA ATT-3'
5'-UUG AUU GCA AAC AAA UGU CCA-3') randomly. Rat models of right MCAO were established according to the method from Taniguchi *et al* [4]. Liposomes containing the less effective shRNA-aquaporin-4 and the more effective siRNA-aquaporin-4 were developed according to a previously described method[29]. In accordance with the method from Bao *et al* [30], prior to MCAO, 5 μL of solution (shRNA-aquaporin-4 or siRNA-aquaporin-4) was injected into the right basal ganglia in the ischemia group. Identically, prior to MCAO, shRNA-containing liposome solution (1:800) and siRNA-aquaporin-4-containing liposome solution (1:800) were injected in the control group and RNA interference group, respectively. Each of the above mentioned groups was further divided into six subgroups as per the time points (0.25, 0.5, 1, 2, 4 and 6 hours), with six rats in each subgroup.

MRI scan

All rats were scanned on a Signa HDx 3.0 T MR scanner (General Electric, Milwaukee, WI, USA) with a rat-specific phased array coil (5 cm in diameter) (Shanghai Chengguang Medical Technology Co., Ltd., Shanghai, China). Animals were placed in a supine position and subjected to coronal T1 weighted imaging scanning, taking the optic chiasm as the center line with a slice thickness of 2 mm, spacing 0 mm, field of view 4 cm × 4 cm, matrix 128 × 128. DW-MRI parameters included an echo planar imaging sequence, repetition time of 9 000 ms, echo time of 102 ms, *b* value of 0 s/mm^2 and 800 s/mm^2.
Apparent diffusion coefficient and relative apparent diffusion coefficient were calculated with the following formulas: apparent diffusion coefficient = In (S_1/S_0) / (b_0–b_1)[31], where S_1 is the signal strength of b = 1 000, S_0 is the signal strength of b = 0, *In* is the natural logarithm; the relative apparent diffusion coefficient = (ischemic apparent diffusion coefficient/contralateral mirror apparent diffusion coefficient) × 100%; measuring the right basal ganglia relative apparent diffusion coefficient. After the MRI scan, rats were sacrificed with an over-dose of 1% (v/v) pentobarbital sodium (30 mg/kg). The brains were fixed with 4% (w/v) paraformaldehyde through cardiac perfusion. The brains were removed by craniotomy for pathological observation and molecular biological experiments.

Pathological observation

The brain tissues containing the right basal ganglia (infarct site) were embedded in paraffin, sliced into 6 μm-thick sections, routinely stained with hematoxylin-eosin, and photographed under the optical microscope (Olympus, Tokyo, Japan). After conventional preparation and double staining using lead and uranium, the samples were observed and photographed under a transmission electron microscope (PECNAL G2F20, Charleston, SC, USA).

Measurement of aquaporin-4 mRNA expression using real time-PCR

Total RNA of the right basal ganglia was extracted

according to the instructions for the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase kit (MBI, Fermentas, Lithuania), and the products were amplified using FTC-2000 quantitative fluorescent PCR equipment (7700, Applied Biosystems, Foster City, CA, USA), with an aquaporin-4 primer: upstream 5'-CAT TTG TTT GCA ATC AAT TAT AC-3', downstream 5'-GAC AGA AGA CAT ACT CGT AAA GT-3'; the length of the product was 214 bp. The aquaporin-4 probe was 5'-CCA GCT CGA TCC TTT GGC-3', and the length of the product was 168 bp. For quantification of mRNA expression by PCR, the dynamic amplification curve was drawn to determine the amplification cycle number (Ct value). According to the formula: $A/B = Ex^{-\Delta\Delta Ct}$, the Ct value was converted to relative aquaporin-4 mRNA expression ($Ex^{-\Delta\Delta Ct}$), where Ct = the fluorescence intensity, *A* = the aquaporin-4 mRNA expression level of sample one and *B* = the aquaporin-4 mRNA expression level of sample two.

Measurement of aquaporin-4 protein expression by western blot analysis

The tissue sample containing the ischemia/reperfusion area identified by DWI was washed twice with PBS, lysed with 200 μL cell lysis buffer (50 mM Tris-HCl at pH 7.6, 5 mM EDTA, 25% (w/v) sucrose, 0.6 μL 100 μg/mL phenylmethylsulfonyl fluoride, 2.4 μL β-mercaptoethanol) for 30 minutes, then centrifuged at 90 000 r/min for 15 minutes at 4 °C. Whole cell extracts equivalent to 100 μg protein were separated using an 8% (w/v) SDS polyacrylamide gel, and then transferred onto nitrocellulose membranes (Gibco, New York, NY, USA). The membranes were blocked with 5% (w/v) skim milk in Tris buffered saline containing Tween-20 (20 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 0.05% (v/v) Tween 20) at 4 °C overnight, followed by incubation with 1:200 mouse monoclonal anti-aquaporin-4 antibody (Santa Cruz Biotechnology) for 2 hours, then incubated for 1 hour at room temperature with 1:10 000 peroxidase-conjugated goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, China). The bound antibody was detected with chemiluminescence (SuperSignal West Pico Stable Peroxide solution (Pierce, Rockford, IL, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as the internal standard. The absorbance ratio of target protein to β-actin was calculated with the Bio-Rad imaging system as the relative expression level of the protein of interest.

Statistical analysis

All measurements were expressed as mean ± SD and were statistically processed using SPSS13.0 software (SPSS, Chicago, IL, USA). Multivariate repeated-measures analysis of variance was performed to compare differences among various time points in the three groups. The correlation between aquaporin-4 mRNA expression and relative apparent diffusion coefficient was analyzed by Pearson's correlation coefficient (α = 0.05).

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Author contributions: Hui Hu was responsible for study assessment, manuscript authorization and funding. Hong Lu had full access to the study concept and design, and was responsible for obtaining funds. Zhanping He and Xiangjun Han were in charge of animal experiments, in particular pathological and molecular biological experiments. Jing Chen and Rong Tu participated in the diffusion-weighted MRI experiments.

Conflicts of interest: None declared.

Ethical approval: All experimental protocols regarding the use of animals in the study were approved by the Institutional Animal Care and Use Committee of Huaxi Medical Center, Sichuan University, China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

REFERENCES

[1] Fisher M, Schaebitz W. An overview of acute stroke therapy: past, present, and future. Arch Intern Med. 2000;160(21):3196-3206.

[2] Kanekar SG, Zacharia T, Roller R. Imaging of stroke: Part 2, Pathophysiology at the molecular and cellular levels and corresponding imaging changes. AJR Am J Roentgenol. 2012;198(1):63-74.

[3] Hofmeijer J, van Putten MJ. Ischemic cerebral damage: an appraisal of synaptic failure. Stroke. 2012;43(2): 607-615.

[4] Taniguchi M, Yamashita T, Kumura E, et al. Induction of aquaporin-4 water channel mRNA after focal cerebral ischemia in rat. Brain Res Mol Brain Res. 2000;78(1-2): 131-137.

[5] Lu H, Hu H, He ZP. Reperfusion of the rat brain tissues following acute ischemia: the correlation among diffusion-weighted imaging, histopathology, and aquaporin-4 expression. Chin Med J. 2011;124(19): 3148-3153.

[6] Gonen KA, Simsek MM. Diffusion weighted imaging and estimation of prognosis using apparent diffusion coefficient measurements in ischemic stroke. Eur J Radiol. 2010;76 (2):157-161.
[7] Lu H, Zhang SZ, Hu H, et al. The relationship between MR diffusion imaging and AQP4 expression in ischemia reperfusion rat model. Zhonghua Fangshexue Zazhi. 2009;43(12):1319-1321.
[8] Rao KV, Reddy PV, Curtis KM, et al. Aquaporin-4 expression in cultured astrocytes after fluid percussion injury. J Neurotrauma. 2011;28(3):371-381.
[9] Gosselin N, Bottari C, Chen JK, et al. Electrophysiology and functional MRI in post-acute mild traumatic brain injury. J Neurotrauma. 2011;28(3):329-341.
[10] van Pul C, Buijs J, Janssen MJ, et al. Selecting the best index for following the temporal evolution of apparent diffusion coefficient and diffusion anisotropy after hypoxic-ischemic white matter injury in neonates. AJNR Am J Neuroradiol. 2005;26(3):469-481.
[11] Ward P, Counsell S, Allsop J, et al. Reduced fractional anisotropy on diffusion tensor magnetic regoilance imaging after hypoxic-ischemic encephalopathy. Pediatrics. 2008;117(4):el-12.
[12] Nakada T. Diffusion tensor analysis: principles and applications. No To Shinkei. 2002;54(4):290-297.
[13] Schellinger PD, Bryan RN, Caplan LR, et al. Therapeutics, and of the American Academy of Neurology TAS. Evidence-based guideline: The role of diffusion and perfusion MRI for the diagnosis of acute ischemic stroke: report of the therapeutics and technology assessment subcommittee of the American Academy of Neurology. Neurology. 2010 ;75 (2):177-185.
[14] Burdette JH, Ricci PE, Petitti N, et al. Cerebral infarction: time course of signal intensity changes on diffusion-weighted MR images. AJR. 1998;171(3):791-795.
[15] Pan J, Konstas AA, Bateman B, et al. Reperfusion injury following cerebral ischemia: pathophysiology, MR imaging, and potential therapies. Neuroradiology. 2007;49(1): 93-102.
[16] Huisman TA, Hawighorst H, Benoit CH, et al. Diffusion weighted MRI: ischemic and traumatic injuries of the central nervous system. Radiology. 2001;41(12): 1038-1047.
[17] Badaut J, Lasbennes F, Magistretti PJ, et al. Aquaporins in brain: distribution, physiology, and pathophysiology. J Cereb Blood Flow Metab. 2002;22(4):367-378.
[18] Lu H, Zhang SZ, Hu H, et al. The relationship between aquaporin-4 expression and apparent diffusion coefficient on diffusion-weighted imaging ischemia reperfusion brain tissues in rats. Int J Cerebrovasc Dis. 2009;17(3): 166-170 .
[19] Tourdias T, Dragonu I, Fushimi Y, et al. Aquaporin 4 correlates with apparent diffusion coefficient and hydrocephalus severity in the rat brain: a combined MRI-histological study. Neuroimage. 2009;47(2):659-666.
[20] Chu K, Kang DW, Kim HJ, et al. Diffusion-weighted imaging abnormalities in wernicke encephalopathy: reversible cytotoxic edema? Arch Neurol. 2002;59(1): 123-127.
[21] Butcher K, Emery D. Acute stroke imaging. Part II: The ischemic penumbra. Can J Neurol Sci. 2010;37(1):17-27.
[22] Sobue K, Asai K, Katsuya H. Aquaporin water channels in the brain and molecular mechanisms of brain edema. Nihon Rinsho. 2006;64(6):1181-1189.
[23] Fazzina G, Amorini AM, Marmarou CR, et al. The protein kinase C activator phorbol myristate acetate decreases brain edema by aquaporin 4 downregulation after middle cerebral artery occlusion in the rat. J Neurotrauma. 2010; 27(2):453-461.
[24] Badaut J, Ashwal S, Adami A, et al. Brain water mobility decreases after astrocytic aquaporin-4 inhibition using RNA interference. J Cereb Blood Flow Metab. 2011;31(3): 819-831.
[25] Yao X, Hrabetová S, Nicholson C, et al. Aquaporin-4-deficient mice have increased extracellular space without tortuosity change. J Neurosci 2008;28:5460-5464.
[26] Nicchia GP, Frigeri A, Liuzzi GM, et al. Inhibition of AQP4 expression in astrocytes by RNAi determines alterations in cell morphology, growth, and water transport and induces changes in ischemia related genes. FASEB J. 2003;17:1508-1510.
[27] Li YH, Sun SQ. Changes of astrocytes AQP4 protein expression during hypotonic medium in vitro. Zhongguo Jijiu Yixue. 2004;24(2):87-90.
[28] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[29] Hu H, Lu H, He ZP. AQP4 expression in hypoxia cultured astrocytes can be inhibited by AQP4 mRNA silence in vitro. Jiepouxue Zazhi. 2011;34(1):73-77.
[30] Bao XM, Shu SY, Li SX, et al. The expression of c-fos proto-oncogene albumin in the brain striatum and the limbic system following injection of kainic acid into Meynert basal nucleus in rats. Diyi Junyi Daxue Xuebao. 1999;19(3):193-196.
[31] Bitzer M, Klose U, Geist Barth B, et al. Alterations in diffusion and perfusion in the pathogenesis of peritumoral brain edema in meningiomas. Eur Radiol. 2002;12(8): 2062-2076.

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