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RESEARCH ARTICLE

Aquaporin-4 gene silencing protects injured neurons after early cerebral infarction

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Abstract

Aquaporin-4 regulates water molecule channels and is important in tissue regulation and water transportation in the brain. Upregulation of aquaporin-4 expression is closely related to cellular edema after early cerebral infarction. Cellular edema and aquaporin-4 expression can be determined by measuring cerebral infarct area and apparent diffusion coefficient using diffusion-weighted imaging (DWI). We examined the effects of silencing aquaporin-4 on cerebral infarction. Rat models of cerebral infarction were established by occlusion of the right middle cerebral artery and siRNA-aquaporin-4 was immediately injected *via* the right basal ganglia. In control animals, the area of high signal intensity and relative apparent diffusion coefficient value on T2-weighted imaging (T2WI) and DWI gradually increased within 0.5–6 hours after cerebral infarction. After aquaporin-4 gene silencing, the area of high signal intensity on T2WI and DWI reduced, relative apparent diffusion coefficient value was increased, and cellular edema was obviously alleviated. At 6 hours after cerebral infarction, the apparent diffusion coefficient value was similar between treatment and model groups, but angioedema was still obvious in the treatment group. These results indicate that aquaporin-4 gene silencing can effectively relieve cellular edema after early cerebral infarction; and when conducted accurately and on time, the diffusion coefficient value and the area of high signal intensity on T2WI and DWI can reflect therapeutic effects of aquaporin-4 gene silencing on cellular edema.

***Key Words:** nerve regeneration; middle cerebral artery occlusion; cerebral ischemia; cytotoxic edema; angioedema; magnetic resonance imaging; diffusion-weighted imaging; aquaporin-4; gene silencing*

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Introduction

Complications of cerebral infarction such as high disability rate and high mortality rate threaten human health and influence a patient's quality of life (Wang and Hu, 2013). Therefore, it is very important to establish a powerful method of treatment and find a tool to identify the effectiveness of this treatment in early cerebral infarction or provide valuable pathological evidence for treatment of this disease.

High-signal intensity on T2-weighted imaging (T2WI) reflects an increase in total water content and represents extracellular edema and necrosis (Hergan et al., 2002). Diffusion-weighted imaging (DWI) is based upon the random Brownian motion of water molecules within a voxel of tissue, and reflects diffusion of water molecules *in vivo*. Nearly all brain disorders can lead to edema and change the diffusion of water molecules. Imaging studies of the disease provide information at a microscopic molecular level using DWI (Schaefer et al., 2005). Apparent diffusion coefficient (ADC) comprehensively reflects activity of free hydrogen ions in living tissue, but can be affected by temperature, interstitial fluid viscosity, pH value, and mainly tissue space (Xu et al., 2010). When tissue space becomes large, hydrogen ions easily diffuse.

Aquaporin-4 (AQP4) belongs to the aquaporin family and is highly expressed in astrocytic foot processes in the brain. AQP4 regulates water molecule channels and the balance of intracellular K^+ concentration (Grange-Messent et al., 1996). AQP4 is an important molecular component of tissue regulation and water transportation in the brain. Because of its polarity of distribution, AQP4 induces bidirectional transport of water molecules along the osmotic pressure gradient (Zhao et al., 2005). After brain injury, a large amount of aberrant water movement, normally mediated by AQP4, leads to the formation of cytotoxic edema (Lu et al., 2004). Simultaneously, AQP4 promotes the reabsorption of excessive water during angioedema, contributes to the scavenging of redundant water in the brain, and then lessens angioedema (Nag et al., 2009; Hergan et al., 2002). Pathological changes in early cerebral infarction (less than 2 hours) include cytotoxic edema in rat models of middle cerebral artery occlusion

(MCAO). High expression of AQP4 has been positively associated with the degree of edema (Lu and Sun, 2003).

The main pathological change in early cerebral infarction is cytotoxic edema, which is also the pathological basis of the ischemic penumbra. An upregulation of AQP4 expression is strongly associated with the degree of cytotoxic edema (Bloch and Manley, 2007; Papadopoulos and Verkman, 2007; Li et al., 2013). Magnetic resonance reflects pathological changes in brain edema. In particular, DWI exactly reveals cytotoxic edema, and ADC negatively correlates with AQP4 expression (Grange-Messent et al., 1996). In relation to the above findings, we investigated whether (i) AQP4 is a key molecular mechanism that leads to early ischemic cytotoxic edema, (ii) AQP4 gene silencing using RNA interference has beneficial effects on early cerebral infarction, (iii) magnetic resonance imaging (MRI) reflects brain changes in the treatment of early cerebral infarction, and (iv) pathological changes in cerebral infarction area after AQP4 gene silencing.

Materials and Methods

Establishment of siRNA-AQP4-MCAO animal models

Ninety healthy Wistar rats of either sex, aged 3–4 months, weighing 300–350 g, were included in this study (license No. SCXK (Chuan) 2008-24; Laboratory Animal Center, West China Medical Center, Sichuan University, China). After fasting for 24 hours before surgery, rats were randomly and equally assigned to model (MCAO), control (shRNA-AQP4 + MCAO) and treatment (siRNA-AQP4 + MCAO) groups. In accordance with the Masuda method (1987), MCAO was performed by inserting a piece of fishing line into the internal carotid artery at a length of 2.5–3.0 cm. Insertion of fishing line, followed by circling after recovery of consciousness indicated success of MCAO induction. Antisense shRNA-AQP4 and siRNA-AQP4 liposomes with the highest interference efficiency were constructed in accordance with Lu et al. (2012). In the control and treatment groups, 5 μL of siRNA-AQP4 solution containing antisense shRNA-AQP4 and siRNA-AQP4 liposomes (1:800) was injected into the right basal ganglia before MCAO. The above groups were divided into five subgroups according to time points following MCAO: 0.5, 1, 2, 4 and 6 hours (n = 6 rats/subgroup). The experimental procedure was approved by the Animal Ethics Committee of Xiangya School of Medicine, Central South University in China.

MRI scan

At 0.5, 1, 2, 4 and 6 hours after MCAO, MRI scan was conducted on a GE Signa HDx 3.0 T MRI system with phased-array coils (Shanghai Chenguang Medical Technologies Co., Ltd., Shanghai, China; rats, 5 cm aperture). Rats were fixed in the supine position, and underwent a T2WI coronal scan centered in the optic chiasm. The following parameters were used: T2WI: repetition time 2,000 ms, echo time 80 ms, slice thickness 2 mm, interslice gap 0 mm, field of view 4 cm × 4 cm, matrix 128 × 128; T2-FLAIR: repetition time 10,000 ms, echo time 100 ms, slice thickness 2 mm, interslice gap 0 mm, field of view 4 cm × 4 cm, matrix 128 × 128; DWI: echo planar imaging, repetition time 9,000 ms, echo time 102 ms, b value 0 s/mm^2 and 800 s/mm^2. The above scans were performed in each group.

Calculation formula:

rs-T2WI = Maximum area of high signal in infarction area in T2WI/ ipsilateral hemisphere area × 100% (rs = relative to muscle);

rs-DWI = Maximum area of high signal in infarction area in DWI/ipsilateral hemisphere area × 100% (rs = relative to muscle);

rADC = ADC in infarction area in DWI/ADC in contralateral mirror area × 100%;

ADC = In($S1$/$S0$)/($b0$–$b1$).

After MRI scan, rats were sacrificed by anesthesia with 1% sodium pentobarbital (30 mg/kg), and fixed with 4% paraformaldehyde injected *via* the heart. Brain tissue for pathological observation was collected by craniotomy.

Morphological observation

The right basal ganglia were embedded in paraffin, and sliced into 6 μm thick sections. Tissue was then stained with hematoxylin and eosin and observed under the light microscope (Olympus, Tokyo, Japan). Samples were prepared under a transmission electron microscope and underwent lead-uranium double staining, whereupon they were observed and photographed using the transmission electron microscope (TEM, H-500, USA).

Statistical analysis

Data of rs-T2WI, rs-DWI and relative ADC are expressed as the mean ± SD, and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Repeated-measures analysis of variance was conducted. A value of $P < 0.05$ was considered statistically significant.

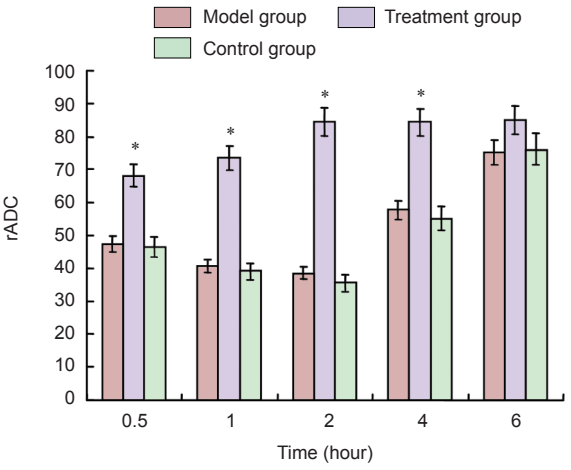

Figure 2 Aquaporin-4 (AQP4) gene silencing on relative apparent diffusion coefficient (rADC) values in MCAO rats.

The rADC changes were identical between the control and model groups. rADC values were significantly higher in the treatment group (AQP4 gene silencing) than in the control group (shRNA-AQP4 + MCAO) at 0.5, 1, 2 and 4 hours (*$P < 0.05$), but no significant difference was detected at 6 hours after MCAO. Data are expressed as the mean ± SD (n = 6 rats/time point, repeated-measures analysis of variance). MCAO: Middle cerebral artery occlusion.

Table 1 Comparisons of T2WI and DWI in the model (MCAO) group at different time points

	Time after MCAO (hour)				
	0.5	1	2	4	6
rs-DWI	21.45±5.40	37.68±5.17	50.72±7.95	81.41±5.03	96.67±5.27
rs-T2WI	0*	0*	21.85±3.80*	64.24±4.62*	93.46±4.71

rs-DWI was larger than rs-T2WI at 0.5, 1, 2 and 4 hours (*$P < 0.05$), but not at 6 hours after middle cerebral artery occlusion (MCAO; $P > 0.05$). Data are expressed as the mean ± SD ($n = 6$ rats/time point, repeated-measures analysis of variance). rs: Relative to muscle; T2WI: T2-weighted imaging; DWI: diffusion-weighted imaging.

Table 2 Comparisons of rs-DWI and rs-T2WI between treatment (AQP4 gene silencing + MCAO) and control (shRNA-AQP4 + MCAO) groups

		Time after MCAO (hour)				
	Group	0.5	1	2	4	6
rs-DWI	Control	21.45±5.40	37.68±5.17	50.72±7.95	81.41±5.03	96.67±5.27
	Treatment	11.25±4.21*	18.53±6.40*	21.45±3.50*	46.84±2.49*	92.08±7.13
rs-T2WI	Control	0	0	22.65±3.20	68.30±5.02	90.46±4.71
	Treatment	0	0	21.45±4.44	70.44±5.49	86.48±7.13

*$P < 0.05$, *vs.* control group. Data are expressed as the mean ± SD ($n = 6$ rats/time point, repeated-measures analysis of variance). rs: Relative to muscle; DWI: diffusion-weighted imaging; T2WI: T2-weighted imaging; MCAO: middle cerebral artery occlusion.

Results

rs-T2WI, rs-DWI and relative ADC values following AQP4 gene silencing in MCAO rats

In the control group, high signal intensity on DWI was observed at 0.5 hours after MCAO. High signal intensity on T2WI was observed at 2 hours and rs-DWI and rs-T2WI increased with time. rs-DWI was consistently larger than rs-T2WI at all time points with the exception of 6 hours ($P < 0.05$; **Figure 1**, **Table 1**). The changes in ADC were identical between control and model groups ($P > 0.05$). Relative ADC values were significantly higher in the treatment group than in the control group at 0.5, 1, 2 and 4 hours after MCAO ($P < 0.05$), but no significant difference was detected at 6 hours after MCAO ($P > 0.05$) (**Figure 2**). rs-DWI was significantly lower in the treatment group than in the control group at all time points with the exception of 6 hours ($P < 0.05$); **Figure 3**, **Table 2**). There was no significant change in rs-T2WI between treatment and control groups at each time point ($P > 0.05$; **Figure 4**, **Table 2**). Relative ADC was on the rise at 0.5–6 hours in the treatment group. There were significant changes in relative ADC values between the treatment and control groups at 0.5–4 hours after MCAO ($P < 0.05$). However, there was no significant difference in relative ADC values between treatment and control groups at 6 hours ($P > 0.05$; **Figure 2**).

Pathological changes following AQP4 gene silencing in MCAO rats

The pathological changes between control and model groups were similar. A few swollen glial cells were seen under the light microscope in the ischemic region in the control group 0.5 hours after MCAO. Eosinophilic staining in the cytoplasm became lighter at this time point, whilst cells increased in size and became round. Under the electron microscope, mitochondrial swelling was observed along with crest deformation, endoplasmic reticulum expansion, nuclear enlargement, and chromatin margination (**Figure 5**). At 1 hour after MCAO, apparent edema was visible. Nuclei were darkly stained and eosinophilic cytoplasm was observed under the light microscope. Cell organelle swelling was observed, but cell membranes were complete under the electron microscope. At 2 hours after MCAO, an increase in cellular edema was observed. Furthermore, pyknosis and evident glial cell swelling deformation were detected, and a photic zone appeared surrounding the cells. Intercellular space was reduced, but cell membranes were complete. At 4 hours after MCAO, angioedema was detectable. Vascular endothelial cell swelling, vascular and intercellular space expansion and vascular deformation were observed. A light red net was visible among tissues. Blood-brain barrier damage was observed under the electron microscope (**Figure 6**). At 6 hours after MCAO, angioedema evidently worsened under the light microscope. Glial cell edema noticeably lessened at 0.5–4 hours after MCAO, especially within 2 hours after MCAO in the treatment group (**Figure 7**). No observable difference in angioedema was detectable in the treatment group at 6 hours after MCAO (**Figure 8**).

Discussion

AQP4 is highly selective towards water molecules in a bi-directional and voltage-insensitive fashion (Jung et al., 1994; Meinild et al., 1998; Fenton et al., 2010). After brain injury, a large amount of aberrant water movement, normally mediated by AQP4, leads to the formation of cytotoxic edema (Lu and Sun, 2003). A high expression of AQP4 has been positively associated with the degree of edema (Grange-Messent et al., 1996). In this study, AQP4 gene silencing had obvious therapeutic effects on cytotoxic edema in the infarct region

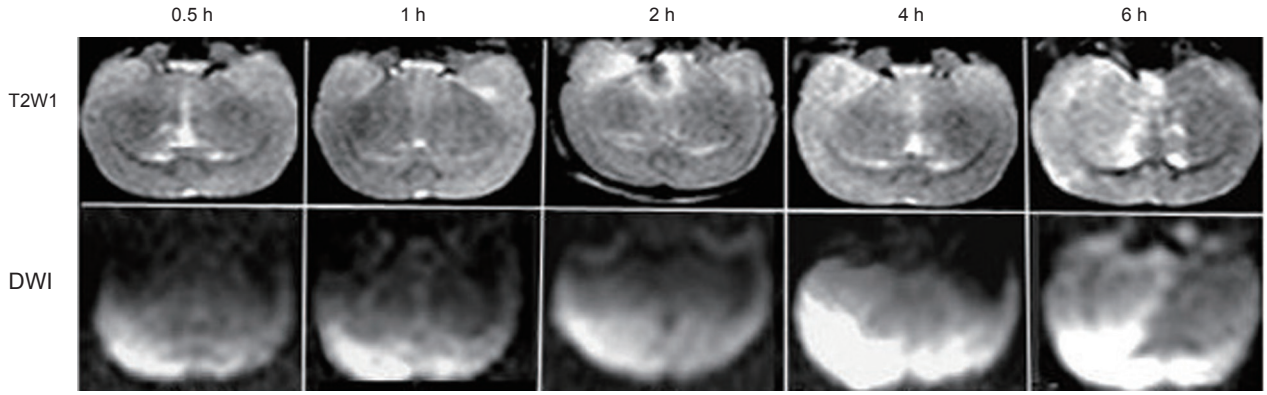

Figure 1 Comparisons of T2WI and DWI in the control (shRNA-AQP4 + MCAO) group.
High signal intensity on DWI appeared at 0.5 hours (h), and high signal intensity on T2WI appeared at 2 h after MCAO. rs-DWI was larger than rs-T2WI at all time points with the exception of 6 hours. rs: Relative to muscle; T2WI: T2-weighted imaging; DWI: diffusion-weighted imaging; AQP4: aquaporin-4; MCAO: middle cerebral artery occlusion.

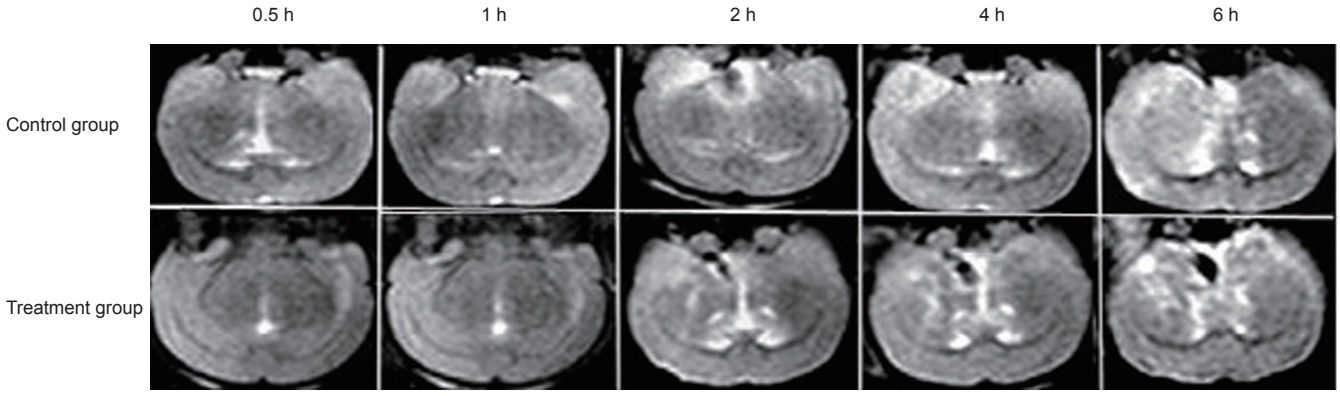

Figure 3 Comparison of rs-DWI between the treatment (AQP4 gene silencing + MCAO) and control (shRNA-AQP4 + MCAO) groups.
rs-DWI was lower in the treatment group than in the control group at 0.5, 1, 2 and 4 hours (h), but not 6 h, after middle cerebral artery occlusion (MCAO). Rs: Relative to muscle; DWI: diffusion-weighted imaging; AQP4: aquaporin-4.

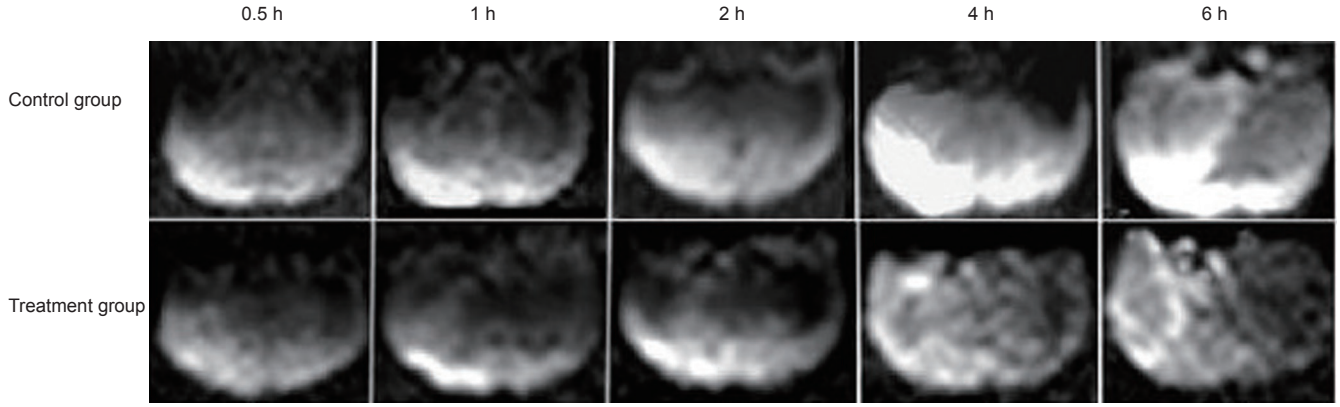

Figure 4 Comparison of rs-T2WI between the treatment (AQP4 gene silencing + MCAO) and control (shRNA-AQP4 + MCAO) groups.
rs-T2WI was similar at 0.5, 1, 2, 4 and 6 hours (h) after middle cerebral artery occlusion (MCAO). Rs: Relative to muscle; T2WI: T2-weighted imaging; AQP4: aquaporin-4.

after early cerebral infarction (less than 2 hours), *i.e.*, the extent of edema was apparently reduced. However, AQP4 did not remarkably improve angioedema in the late stage (6 hours) after cerebral infarction. Results from this study showed that siRNA-AQP4 directly decomposed AQP4 gene fragments, reduced AQP4 protein synthesis, and decreased the metastasis of hydrogen ions outside cells into cells. During mixed edema, blood-brain barrier damage did not lessen with the reduction in AQP4 protein. Therefore, high AQP4 expression is a key molecular mechanism underlying cytotoxic edema during early cerebral infarction and is not obviously correlated with blood-brain barrier damage

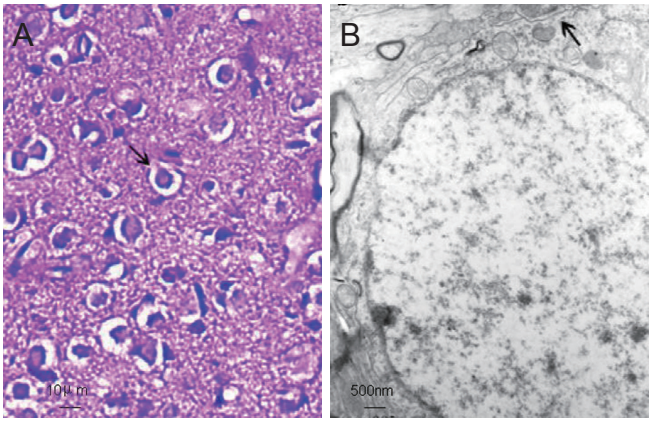

Figure 5 Cytotoxic edema.
(A) A few swollen glial cells (arrow) were visible under the light microscope in the ischemic region in the control group (shRNA-AQP4 + MCAO) at 0.5 hours after MCAO. Eosinophilic staining in the cytoplasm became lighter at this time point, whilst cells increased in size and became round (hematoxylin-eosin staining, × 200). (B) Mitochondrial swelling (arrow) was observed along with crest deformation, endoplasmic reticulum expansion, nuclear enlargement, and chromatin margination (transmission electron microscopy, × 6,000, arrow). AQP4: Aquaporin-4; MCAO: middle cerebral artery occlusion.

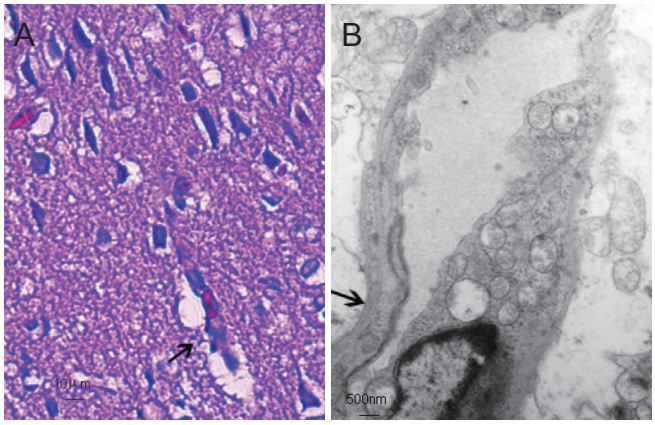

Figure 6 Angioedema was detectable.
(A, B) Vascular endothelial cell swelling (A, arrow), vascular and intercellular space expansion, and vascular deformation (A, arrow) were observed in the control group (shRNA-AQP4 + MCAO) at 4 hours after MCAO (hematoxylin-eosin staining, × 200), as was blood-brain barrier damage (B, transmission electron microscopy, × 6,000, arrow). AQP4: aquaporin-4; MCAO: middle cerebral artery occlusion.

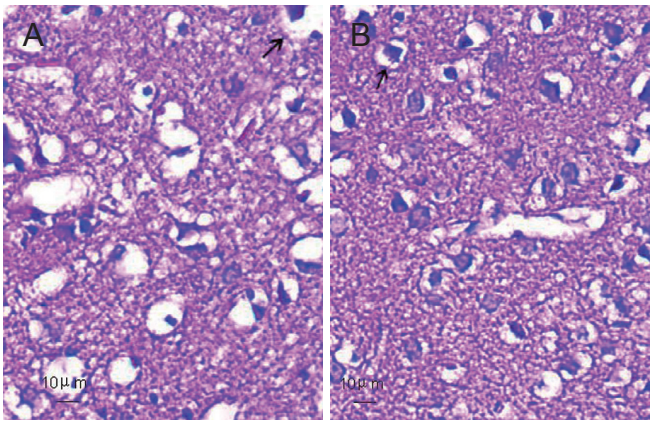

Figure 7 Cytotoxic edema (hematoxylin-eosin staining, × 200).
Compared with the control group (shRNA-AQP4 + MCAO; A), cytotoxic edema was obviously reduced in the infarct region at 2 hours after MCAO in the treatment group (MQP4 gene silencing + MCAO; B). Arrows show cytotoxic edema. AQP4: Aquaporin-4; MCAO: middle cerebral artery occlusion.

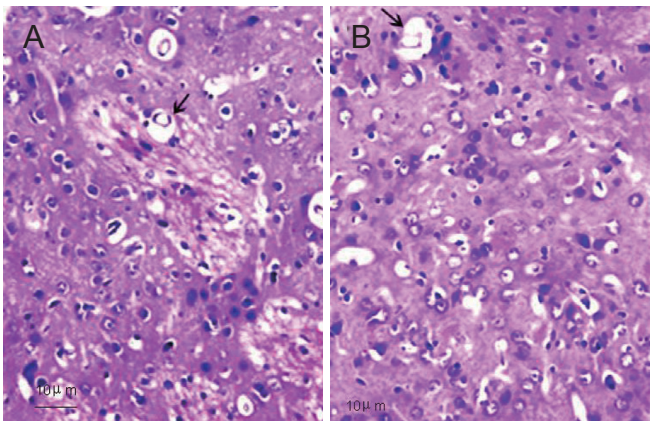

Figure 8 Angioedema (hematoxylin-eosin staining, × 200).
Compared with the control group (shRNA-AQP4 + MCAO; A), angioedema did not greatly change in the infarct region at 6 hours after MCAO in the treatment group (AQP-4 gene silencing + MCAO; B). Arrows show angioedema. AQP4: Aquaporin-4; MCAO: middle cerebral artery occlusion.

during late cerebral infarction.

Notably, significant astrocytic accumulation of cytosolic AQP4 has yet to be demonstrated (Potokar et al., 2013; Assentoft et al., 2014; He et al., 2014). Interestingly, no distinct regulatory pattern in AQP4 expression after MCAO is readily discernible: AQP4 expression was found to be up-regulated (Higashida et al., 2011; Huang et al., 2013; Li et al., 2013) or down-regulated (Friedman et al., 2009; Liu et al., 2010; Shin et al., 2011) in a time-dependent manner (Nito et al., 2012; Zeng et al., 2012). Our previous studies demonstrated that high signal intensity appeared on DWI 15 minutes after MCAO. The area of high signal intensity increased with prolonged time until 2 hours, but ADC values decreased linearly. Pathological changes are mainly cytotoxic edema, but AQP4 expression increased linearly. Therefore, during early cerebral infarction, ADC values were negatively correlated with cytotoxic edema and AQP4 expression (Kleindienst et al., 2006; Lu et al., 2011, 2012). In the present study, high signal intensity on DWI and T2WI appeared at 0.5 and 2 hours separately in the control group. The area of high signal intensity increased gradually with prolonged time. However, the area of high signal intensity on DWI was smaller in the treatment group than in the control group. Nevertheless, angioedema was not apparently relieved, and the area of high signal intensity on T2WI was not obviously reduced. At the same time, relative ADC diminished rapidly, and pathological manifestations were cytotoxic edema at 2 hours. At 4 and 6 hours after MCAO, angioedema was visible. Simultaneous-

ly, ADC values were higher in the treatment group than in the control group at 0.5–4 hours after MCAO. This study demonstrated that cytotoxic edema evidently lessened in the treatment group using AQP4 gene silencing. Overall, the above findings suggested that AQP4 gene silencing effectively lessened cytotoxic edema, but did not exert significant therapeutic effects on angioedema. DWI (ADC) exactly reflects these pathological changes, but T2WI cannot.

In summary, increase in AQP4 expression is a major molecular mechanism underlying early cerebral infarction. AQP4 gene silencing can effectively treat early cerebral infarction and relieve cytotoxic edema. DWI (ADC values) precisely monitors and assesses therapeutic effects. Our results go some way toward providing experimental evidence for drug development and efficacy evaluation using imaging methods in the treatment of early cerebral infarction.

Author contributions: *HL conceived and designed the study and was responsible for this paper. ZPH performed the experiments, provided reagents/materials/analysis tools, and wrote the paper. Both of these two authors analyzed the data and approved the final version of the paper.*

Conflicts of interest: *None declared.*

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