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Original Article

Effect of AQP4 RNAi on apoptosis of cells surrounding hematoma in rat hemorrhage

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Abstract: This study aims to investigate the effect of aquaporin-4 (AQP4) siRNA on cell apoptosis surrounding hematoma in rat hemorrhage models. Total 30 SD rats were divided into 3 groups: hemorrhage model group, blank plasmid group and AQP4 siRNA group, with 10 rats in each group. The caudate putamen brain hemorrhage model was induced by Thrombin VII injection. A modified Longa grading method was applied to classify neurological function. The content of water in brain was determined and TUNEL assay was used to detect apoptosis. AQP4 expression was detected by RT-PCR, and the expressions of matrix metalloprotei-2 (MMP-2), MMP-9, Caspase-3, and Bcl-2 were detected by Western Blot. The estimated scores about neurological function were significantly lower in AQP4 siRNA group than in blank plasmid group and hemorrhage model group ($P < 0.05$). Rat brain tissue bled obviously and hematoma was observed in brain tissues in blank plasmid group and hemorrhage model group. In AQP4 siRNA group, the content of water in brain tissue was about 76.7%. Cell apoptosis surrounding hematoma was less severe in AQP4 siRNA group. AQP4 RNA expression was significantly higher in blank plasmid group and hemorrhage model group. The expressions of MMP-2/MMP-9 and Caspase-3 decreased significantly in AQP4 siRNA group, while Bcl-2 expression significantly increased ($P < 0.05$). AQP4 down-regulation by siRNA can relieve the damage of neurological function damage and represses cell apoptosis around hematoma. These may be associated with the decrease of MMP-2, MMP-9, and Caspase-3, and the increase of Bcl-2.

Keywords: AQP4, RNAi, hemorrhage

Introduction

Cerebral hemorrhage is a common disease in older patients. With high rate of mortality and morbidity, it seriously threats to human life. It is about 20% for the patients dead due to cerebral hemorrhage among all disease mortality [1]. There is no effective prevention and treatment for the damage by brain hemorrhage, and the two types of damages, hematoma occupation effect and directly injuries on surrounding brain tissues, are the two main mechanisms of hemorrhagic brain injury [2-4].

Actually the secondary injury is an important factor to influence the prognosis [2]. It was validated that brain tissue around hematoma had incomplete ischemic area, which resulted in secondary damage by inducing cell apoptosis [5]. Cell apoptosis was involved in the secondary injury of brain hemorrhage. Apoptosis processes included inducible initiation, intracellular regulation, implementation and phagocytosis. One of the features of apoptosis is water loss and cell shrinkage. The water was lost by simple diffusion of hydrophobic phospholipid bilayer or by help from some aquaporins. It was reported that cell apoptosis around hematoma was associated with aquaporins [6-9].

Aquaporin (AQP) is one kind of molecules for water channel. More than 13 kinds of aquaporin (AQP0~AQP12) have been found in mammalian tissues, among which aquaporin 4 (AQP4) expressed richly in brain tissue. AQP4 participated in the processes of the formation and regression of brain edema, which played important regulatory roles in motion of water across the cell membrane during apoptosis [10]. In this study, we tried to down-regulate AQP4 expres-

sion by RNAi technology, which relieved hematoma and apoptosis of brain cells and protected brain tissues surrounding hematoma to reduce secondary damage.

Methods and materials

Construction of AQP4 siRNA plasmid

The sequence of AQP4 full length mRNA was searched from NCBI gene database. Then AQP4 siRNA was designed based on AQP4 coding region by gene silencer software. The target sequence of AQP4 siRNA was 5'-AGA TCA GCA TCG CCA AGT C-3' (Register Number 012825, 326-345 bp in AQP4 mRNA). And the sense strand of target dsRNA was 5'-AGAUCAGCAUCGCCAAGUCUU-3', while the antisense strand was 5'-UUUCUAGUCGUAGCGGUUCAG-3'.

Preparation of intracerebral hemorrhage (ICH) model

Total 30 SD rats were purchased from Sichuan medical university and divided into 3 groups: rat hemorrhage model group, blank plasmid group and AQP4 siRNA group, and each group has 10 rats. ICH model was induced by injection of thrombin VII (sigma, St. Louis, USA) for the caudate putamen. Rats were narcotized by intraperitoneal injection of 3.5% chloral hydrate (10 ml/kg). To be ensured with spontaneous breathing, rats were fixed in three-dimensional positioning system (ZH-B type, Huaibei Zhenghua biometric devices Limited Company, Huaibei, China) to make the skull around the same level. The skull was exposed with 1.5 cm sagittal incision in the middle of the cranial line. Behind of the skull about 1 mm, a 1 mm hole was drilled by screwdriver at 3 mm right side of the sagittal line. Enabling the needle and the hole in the same straight line and the inserted needle in about 6 mm, 0.5 U collagenase VII was injected uniformly for about 5 min, and needle retention kept for 10 min. Then the needle was slowly moved out for 5 min, in total about 20 min. Finally, the cranial pore was blocked by bone wax, after sewing and disinfection, rat was placed separately in empty cage to wake. For the AQP4 siRNA group: 1 μg plasmid containing siRNA was mixed with 100 μL lipofectamine 2000, then the mixture was injected into the window parts of rat skull once every two days. The blank groups were treated similar as siRNA group, only the injected plasmid did not contain AQP4 siRNA fragment.

Evaluation of neurological function

After surgery 24 h, modified Logna classification method was applied to evaluate the behavioral scores in each group. The score criteria was: 0 point, no neurological deficit symptoms; 1 point, cannot extend the contralateral forelimb in surgery; 2 points, contralateral forelimb flexion in surgery; 3 points, slightly circling to the contralateral side in surgery; 4 points, severe circling to the contralateral side in surgery; 5 points, falling to the contralateral side in surgery. Rats scored more than 2 points were regarded as successful experimental model. After 3 days rats were sacrificed for further detection.

Measurement of water content in brain tissue

After 3 days, the model rats were killed and the brain tissues around the hemorrhage were extracted to measure the water content by dry-wet method. About 2 mm thick ipsilateral brain tissue was used to detect the water content after removal of the frontal pole. Firstly, the wet weight of brain tissue was weighed out, expressed as A; Secondly, brain tissue was weighted after wrapped with aluminum foil and baked in the oven 100°C for 24 h, expressed as the dry weight, B. Finally, the water content was calculated according to the formula: brain water content = (B-A)/B × 100%.

Apoptosis of brain tissues by TUNEL assay

In each group, 5 rats were randomly selected for anesthesia by intraperitoneal injection of chloral hydrate. The brain tissue was removed to fix by 4% paraformaldehyde from each rat. After washing with 0.01 M PBS, the tissues were dehydrated in conventional gradient ethanol, xylene, embedded with paraffin. Tissue sections were prepared with a thickness of 5 mm tissue slices by histotome. The apoptosis was detected by the standard protocol of TUNEL apoptosis kit (Nanjing KG Biological Technology Development Co., Ltd., Nanjing, China). Tissue slice was dewaxed by xylene for 5 min 2 times, followed by gradient of ethanol (100% × 5 min, 100% × 3 min, 95% × 3 min, 85% × 3 min, 70% × 3 min, 50% × 3 min) for hydration. After washing by PBS and fixing by 4% paraformaldehyde for 15 min, each slice was incubated with 100 μL 20 μg/mL Proteinase K for about 15 min. After fixing again, each slice was balanced in wet box for

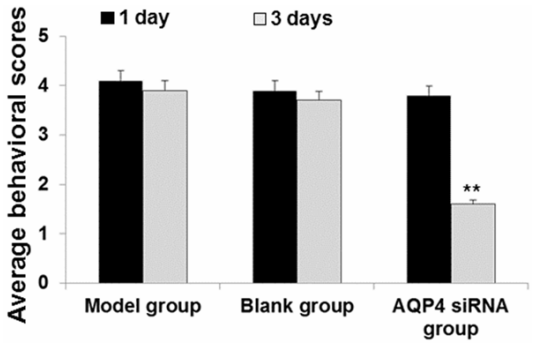

Figure 1. Evaluating the neurological function of rats in different groups by the modified Logna classification method. Compared with model group, $^{**}P < 0.01$.

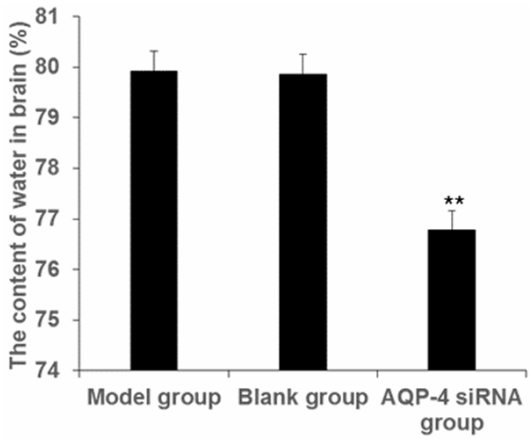

Figure 2. Water content of brain tissues in different groups. Compared with model group, $^{**}P < 0.01$.

10 min. Then 100 μL TUNEL reaction mix was added to each slice and reacted in dark humid chamber at 37°C covered by sealing membrane or coverslip. After Dealt with 2 × SSC 15 min and 0.3% H_2O_2 15 min, 100 μl streptavidin labeled HRP (1:500 PBS dilution) was added for 30 min. DAB was used to color in dark, and hematoxylin was used for staining.

AQP4 expression in brain tissues surrounding hematoma by RT-PCR

Each 1 mL Trizol (Sigma-Aldrich Co. LLC., St. Louis, USA) reagent was added per 100 mg brain tissue after homogenate on ice. Shaking vigorously for 30 s after adding chloroform, the mixture was centrifuged at 12000 g, 4°C for 20 min. The supernatant was transferred to another EP to add equal volume of isopropanol. After mixing and centrifuging at 12000 g, 4°C for 20 min, the supernatant was discarded. After adding 700 μL 75% ethanol, the mixture was centrifuged at 12000 g, 4°C for 10 min. The sediment was dried in the air for 20-30 min, then 20 μL DEPC treated H_2O was added to dissolve. RT-PCR was used for amplification and alpha RED was used for gel image analysis. The PCR reaction conditions were following: 94°C for 5 min, and followed by 32 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, followed by another 10 min at 72°C for extending. The primers of AQP4 were: 5'-CCAGCTGTGATTCCAAAACGGAAC-3' and 5'-TCTAGTCATACTGAAGACAA TACCTC-3', with 305 bp product length. The internal reference was β-actin and the primers were 5'-GCCATGTACGTAGCCATCCA-3' and 5'-GAACCGCTCATT GCCGATAG-3', with 375 bp product length. The PCR conditions were following: 95°C for 2 min, and followed by 32 cycles at 95°C for 30 s, 52°C for 30 s, 72°C for 40 s, followed by another 5 min at 72°C for extending.

MMP-2, MMP-9, Caspase-3 and Bcl-2 expression in brain tissues by Western Blot

Rats were divided into 3 groups: rat hemorrhage model group, blank plasmid group and AQP4 siRNA group. After 3 days, the model rats were killed and the brain tissues around the hemorrhage were extracted to extract total proteins. Each 100 mg tissue was lysed with 200 μL lysis buffer containing 2 μL PMSF and protease inhibitor on ice for 10 min. The lysate was centrifuged at 12000 g for 10 min at 4°C. The protein concentration was detected by BCA method. The proteins was loaded into SDS-PAGE and then transferred to PVDF membrane. The polyclonal antibodies of MMP-2, MMP-9, Caspase-3 and Bcl-2 were rabbit anti-rat. After incubation with antibody overnight, the membrane was washed by TBST. The secondary antibody was HRP-conjugated goat anti-rabbit IgG. All the antibodies were purchased from Abcam Company (Cambridge, England). Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The β-actin was used as an internal control to calculate the relative expression of ISG15. The developed film was scanned and analyzed by Image pro-plus software (Media Cybernetics, Inc., MD Rockville, USA).

Statistical analysis

All the data were shown as the mean ± SD, and difference was determined by ANOVA and SNK

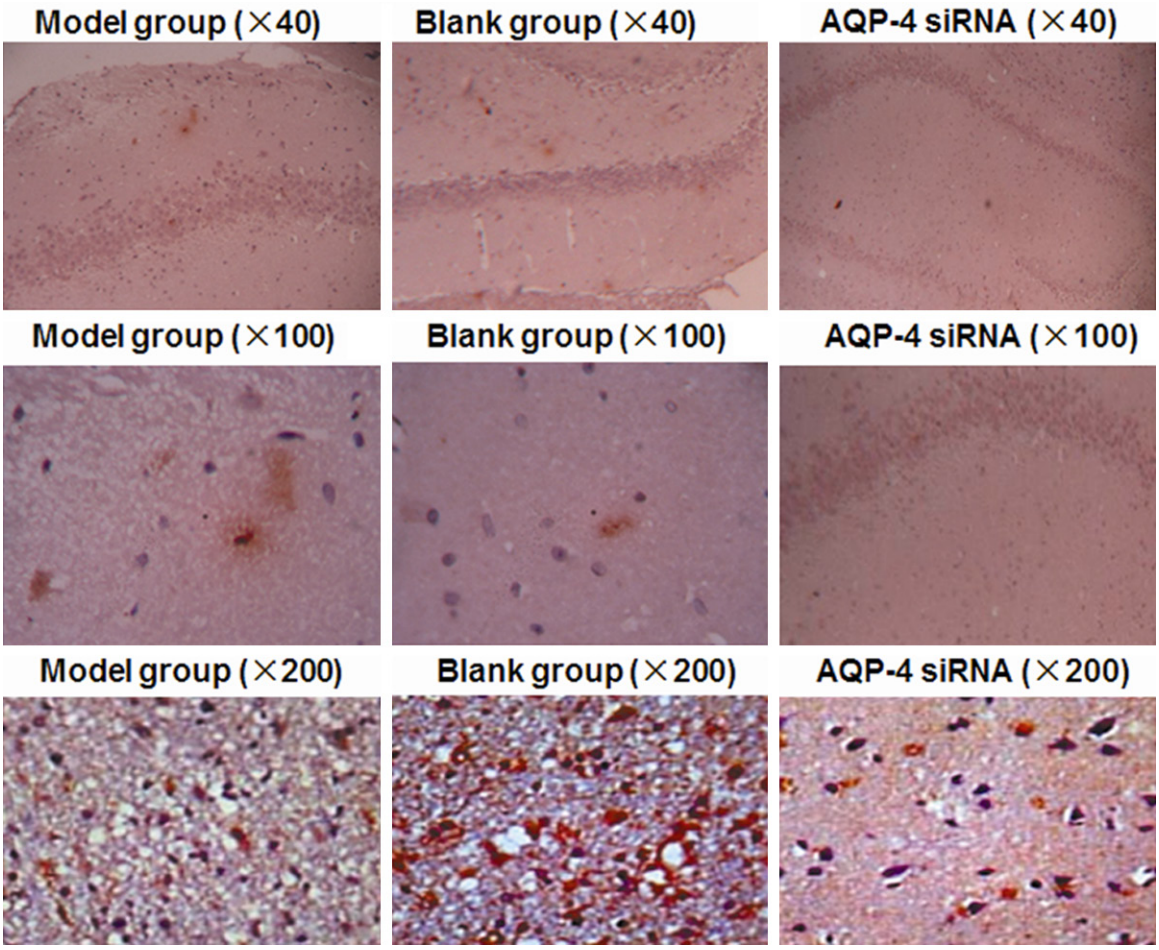

Figure 3. TUNEL method to detect the apoptosis in different groups. AQP4 siRNA group was lighter stained than other groups, which indicated that AQP4 was associated with apoptosis of brain cells. The images at 40 ×, 100 × and 200 × magnification were shown.

method. The SPSS 16.0 software was used to do statistical analysis. $P < 0.05$ was considered as statistically significant.

Results

Scoring of neurological function in different groups

To evaluate the neurological function of rats in different groups, the modified Logna classification method was applied to evaluate the behavioral scores. As shown in **Figure 1**, the results showed that the scores in model group and blank group were higher than in AQP4 siRNA group after 1 day of surgery, although the difference has no significance. After 3 days of surgery, the scores were significantly decreased in AQP4 siRNA group than blank group ($P < 0.01$), while there was no significant difference between model group and blank group.

Measurement of the content of water in brain tissues in different groups

To investigate how AQP4 participated in brain hemorrhage model after interference by siRNA, the dry-wet method was used to detect the water content of brain tissues surrounding hematoma. The results showed that hemorrhage was obvious in brain tissue and surrounding tissue formed significant brain edema. The water content of brain tissues in model group and blank group were 79.9% and 79.8% respectively, while it was 76.7% in AQP4 siRNA group, as shown in **Figure 2**. The difference was statistically significant ($P < 0.01$).

The apoptosis of brain cells surrounding hematoma

To study the influence on apoptosis of brain cells of AQP4, we applied TUNEL method to

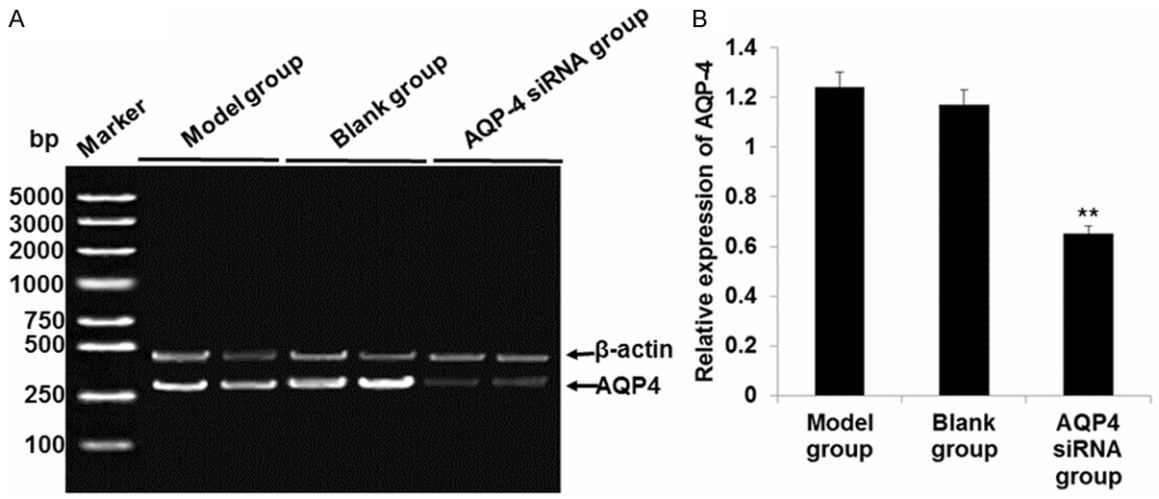

Figure 4. RT-PCR to detect the AQP4 expression in brain tissues after RNA interference. A. The agarose gel was used to detect the amplification of *AQP4* gene in different groups. B. Statistical analysis of the expression of *AQP4* gene in different groups. Compared with model group, $^{**}P < 0.01$.

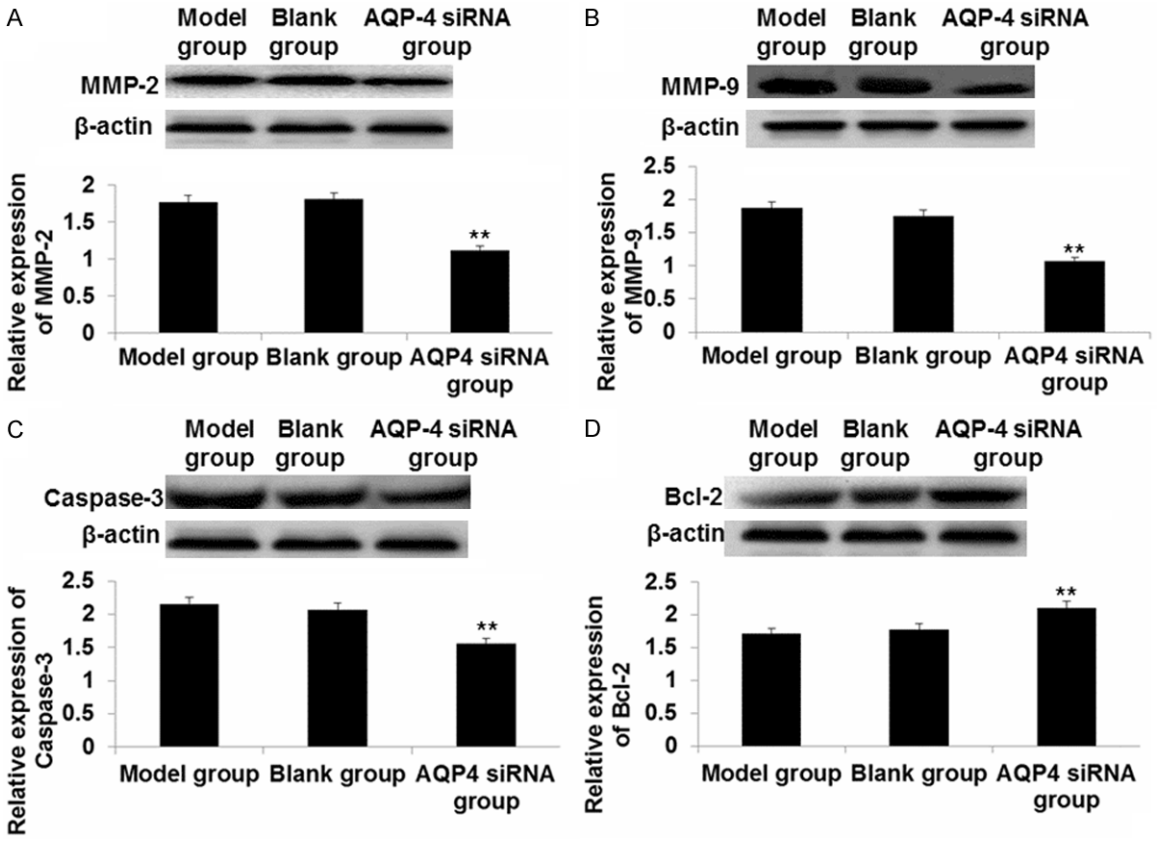

Figure 5. Western Blot to detect the expression of MMP-2, MMP-9, Caspase-3 and Bcl-2 protein in different groups. A. MMP-2 expression was significantly lower in brain tissues surrounding hematoma in AQP4 siRNA group than in either blank group or model group. Compared with model group, $^{**}P < 0.01$. B. MMP-9 expression was significantly lower in brain tissues surrounding hematoma in AQP4 siRNA group than in either blank group or model group. Compared with model group, $^{**}P < 0.01$. C. Caspase-3 expression was significantly lower in brain tissues surrounding hematoma in AQP4 siRNA group than in either blank group or model group. Compared with model group, $^{**}P < 0.01$. D. Bcl-2 expression was significantly higher in brain tissues surrounding hematoma in AQP4 siRNA group than in either blank group or model group. Compared with model group, $^{**}P < 0.01$.

detect the apoptosis in different groups. It was shown that hemorrhage was obvious in model group and blank group, and many regions were stained deeply in brain tissue surrounding hematoma after 3 days of surgery (**Figure 3**). In AQP4 siRNA group, the stained color was light, which indicated that AQP4 was associated with apoptosis of brain cells.

AQP4 expression in brain tissues after RNA interference

To detect the expression changes of AQP4 mRNA after RNA interference, RT-PCR was applied in different groups. As shown in **Figure 4A** and **4B**, AQP4 expression was significantly decreased in AQP4 siRNA group than in model group and blank group ($P < 0.05$), while there was no significant difference between model group and blank group.

Expression of MMP-2, MMP-9, Caspase-3 and Bcl-2 in protein level in brain tissues

In order to detect the expression of extracellular matrix proteins and apoptosis associated proteins, we applied Western Blot to detect the expression of MMP-2, MMP-9, Caspase-3 and Bcl-2 in brain tissues surrounding hematoma in different groups. As shown in **Figure 5A-C**, expressions of MMP-2, MMP-9 and Caspase-3 were all significantly higher in brain tissues surrounding hematoma in blank group and model group than in AQP4 siRNA group ($P < 0.05$). Bcl-2 expression was significantly down-regulated in model group and blank group than in AQP4 siRNA group, as shown in **Figure 5D** ($P < 0.05$). The results indicated that AQP4 regulated the expression of apoptosis associated genes to repress apoptosis of brain cells. Down-regulated AQP4 induced by AQP siRNA can relieve the hematoma bulk and the damages of hemorrhage through regulating MMPs proteins.

Discussion

Intracerebral hemorrhage (ICH) is one of disease with higher mortality, while the mechanisms are not yet clear. Currently the methods used for preparation of ICH animal model included micro balloon expansion, bacterial collagenase injection, autologous blood injection, and spontaneous intracerebral hemorrhage models [11]. In this study, we applied injection of thrombin VII to induce ICH model of caudate putamen of the brain. Bacterial collagenase is one kind of proteolytic enzyme that can degrade the basement membrane and interstitial collagen vascular to destroy cerebral vascular basement membrane, resulting in hemorrhage. It was characterized by slow formation of hematoma by oozing into the interstitial space, and large hematoma was merged gradually from small pieces [12]. The ICH model prepared by collagenase injection method has the advantage of bleeding fast, obvious edema, and low mortality, which is suitable as research model for studying pathological and physiological mechanisms, biochemistry changes, recovery of neurological function, and drug treatment effect [13]. In this study, it was observed that our prepared ICH model was successful after injection 3 days.

Aquaporin is a class of proteins that form transfer channel in all life, which allows the water move between cells and surrounding environments [14]. AQP4 is mainly distributed in membrane or foot process of endothelial cells, gliacytes in capillary, pia mater, and ventricular zone. The distribution of AQP4 in brain indicated that it was associated with the transport of water in brain, which plays important roles in maintaining the water balance in brain [15]. The relationship of AQP4 expression with cerebral hemorrhage is still unclear. Cerebral hemorrhage can increase AQP4 expression and induce apoptosis of nerve cells and brain cells surrounding hematoma [16]. RNA interference technology is a post-transcriptional gene silencing mechanism induced by siRNA, which can repress expression of target genes to further study the associated function [17]. In this study, we constructed AQP4 siRNA vector to regulate AQP4 expression in brain. The results showed that edema was reduced significantly in AQP4 siRNA group than in model group and blank group, which indicated that AQP4 was involved in the regulation of cerebral hemorrhage. In the process of cerebral hemorrhage, cells around hematoma also appeared apoptosis. The apoptosis in model group and blank group was obvious although it was unclear about the relationship of AQP4 with apoptosis associated factors. So in this study, we further investigated whether AQP4 expression can regulate the expression of MMP-2, MMP-9, Caspase-3 and Bcl-2.

MMP-2 and MMP-9 belongs to serum matrix metalloproteinase, which is one of the most important proteases to degrade the extracellular matrix (ECM). MMP proteins can participate varieties of diseases, such as demyelination and Blood Brain Barrier (BBB) injury [18]. In recent years, MMP proteins were found closely related to brain ischemia. In normal brain, MMP-2 and MMP-9 can be secreted by microvascular endothelial cells, and astrocytes may produce inactive MMP-2, while microglia can produce MMP-9 in the presence of inflammatory cytokines [19, 20]. Over-expressed MMP-2 and MMP-9 can be involved in the BBB damage in early stage after bleeding, which promotes formation of vasogenic edema [21]. Through the destruction of ECM, MMP-9 may affect the BBB permeability, which leads to cerebral edema [22]. MMP-9 expression was related with volume of edema around hematoma and deterioration. Thus, over-expressed MMP-9 was usually regarded as a predictor of acute phase of cerebral edema and deterioration [23, 24].

Bcl-2 family is closely related to cell apoptosis, and Bcl-2 mutually opposed to BAX in function, which cooperate to regulate cell apoptosis [25]. As an inhibitor of apoptosis, Bcl-2 was expressed broadly in the central nervous system. Up-regulation of Bcl-2 protein was related to neuronal apoptosis in tissue around the hematoma in ICH patients. The mechanisms were Bcl-2 can repress the release of proteins that can promote apoptosis [26-28]. In early stage of cerebral hemorrhage, pro-apoptotic factor expression increased obviously, such as Caspase family, which can activate specific signaling pathway, produce nuclear shrinkage, and form DNA fragment, then ultimately control the development of apoptosis [29, 30]. Playing direct roles in proteolytic in apoptotic cells, Caspase-3 can lead to functional protein cleavage to result in the occurrence of apoptosis after brain hemorrhage [31]. In cerebral hemorrhage, it can be speculated that anti-apoptotic factor can't neutralize the apoptotic factors to break the balance, then resulting in apoptosis [32].

In this study, it was shown that MMP-2/MMP-9 expression increased significantly in brain tissues of model group and blank group, while they were down-regulated in AQP4 siRNA group. This indicated that AQP4 participated in the regulation of MMP-2/MMP-9 expression in the processes of cerebral hemorrhage. Caspase-3 expression was significantly increased in brain tissues in model group and blank group than in AQP4 siRNA group, while Bcl-2 expression was higher in AQP4 siRNA group than in model group and blank group. These results suggested that the expression of pro-apoptotic factor was higher than anti-apoptotic factor when AQP4 was expressed. It was shown that AQP4 expression was correlation with the expression of MMP-2, MMP-9, Caspase-3 and Bcl-2. Without AQP4 interference, AQP4 can promote expressions of MMP-2, MMP-9 and Caspase-3, while inhibit Bcl-2 expression, which plays important roles in cell apoptosis in cerebral hemorrhage.

In summary, we found that down-regulated AQP4 can reduce brain edema, inhibit apoptosis of cells surrounding hematoma, decrease the expression of pro-apoptotic factor like MMP-2, MMP-9 and Caspase-3, and promote anti-apoptotic factor Bcl-2 expression. These results indicate that AQP4 plays an important role in cerebral hemorrhage, which may be regarded as potential target to cure IHC.

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Disclosure of conflict of interest

None.

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