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Effects of Administering Hirudin During Different Time Windows on Perihematomal Expression of AQP4 and Brain Water Content in Rat Model of Intracerebral Hemorrhage

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ABSTRACT

Background: Recently published studies suggest that reducing the effect of thrombin and AQP-4 using inhibitors or antagonists might help decrease brain edema after Intracerebral hemorrhage (ICH), but the optimal time window for administering a thrombin inhibitor or antagonist is poorly understood. The present study aimed to determine the effect of the direct administration of a thrombin inhibitor (hirudin) to a hematoma at different time points after ICH on perihematomal brain edema and the expression of AQP4. **Materials and Methods:** Forty-eight healthy male Wistar rats were randomly distributed into the model control group (MC group), the 6 h medical treatment group (MT group), the 12 h MT group or the 18 h MT group. A model of ICH was performed through the stereotaxic injection of autologous arterial non-anticoagulant blood into the basal ganglion of the rat brain. Hirudin solution was directly infused into the hematoma cavern 6 h, 12 or 18 h after the successful induction of ICH. All of the rats were sacrificed 48 h after ICH and the brains were collected. The perihematomal BWC was measured using the dry-wet weighing method. RT-PCR was performed to analyze the mRNA expression of AQP4 in the perifocal brain tissues and Western blot was performed to analyze the protein expression of AQP4. **Results:** Compared to the MC group, the mRNA and protein expression levels of AQP4, as well as the BWC, were significantly decreased after hirudin treatment, especially in the 6 and 12 h group ($p<0.05$). **Conclusion:** The direct administration of hirudin to a hematoma cavern could reduce the formation of perifocal brain edema and decrease the mRNA and protein expression of AQP-4 in the brain tissues surrounding the hematoma. The optimal time window for administering hirudin might be within 6-12 h.

Key words: Intracerebral hemorrhage, thrombin inhibitor, AQP4, BWC, time window, hirudin

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INTRODUCTION

Intracerebral hemorrhage (ICH) is the most serious but least treatable form of stroke, displaying a high morbidity and mortality and leaving survivors with severe neurological deficits. The prognosis of patients after ICH critically depends on both the size and the degree of hematoma expansion (Anderson, 2009). The acute treatment of ICH has suffered from a lack of guidance that is based on reliable clinical trial data (Bernstein and Del-Signore, 2005). After the initial hemorrhage, hematoma expansion and perihematomal edema result in secondary brain damage that worsens the outcome (Elliott and Smith, 2010).

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The hallmark of ICH-induced brain injury is BBB disruption, which contributes to edema formation, the influx of leukocytes and the potential entry of neuroactive agents into the perihematomal brain. Brain edema is one of the most frequent and serious complications of ICH. However, the mechanism by which ICH causes brain edema remains unknown (Wu *et al*., 2008). The formation of brain edema has been linked to thrombin toxicity, which is induced by blood clotting during the acute stage of ICH. Thrombin, matrix metalloproteinase-9 (MMP-9) and aquaporin-4 (AQP-4) are stroke-related inflammatory mediators that have been implicated in ICH pathology. These mediators appear very early and persist for several days after ICH and the temporal patterns of expression of thrombin and AQP-4 are associated with brain edema formation after ICH (Wu *et al*., 2010). Lee *et al*. (1995) assessed the effect of

thrombin on the brain parenchyma, revealing that thrombin caused brain edema when infused into the brain at concentrations as low as 1 U μL^{-1} (Lee *et al*., 1995). The same group demonstrated that cell toxicity and BBB disruption induced by thrombin are triggering mechanisms for edema formation following ICH (Lee *et al*., 1997).

AQP-4 and AQP-9 are proteins that are expressed on astrocytes in the rat brain and are involved in the accumulation of water in the brain during brain edema. Autologous whole blood infusion into the caudate nucleus up-regulated the mRNA and protein expression levels of AQP-4 and AQP-9 (Sun *et al*., 2009). An increase in AQP-4 expression following ICH in AQP-4 (+/+) mice was observed and the up-regulation of the expression of both thrombin and AQP-4 is associated with brain edema formation after ICH. Secondary edema and brain damage may correlate with the mRNA expression of AQP-4 in the perihematomal area of brain edema; removal of the hematoma helps decrease the AQP-4 mRNA expression level and brain edema-induced damage during the early stage of ICH (Guo *et al*., 2008). However, some experimental studies reached contrary conclusions (Tait *et al*., 2010; Tang *et al*., 2010). Thus, the effect of AQP-4 in the formation of brain edema after ICH remains unclear. Thrombin and AQP-4 levels increased to aggravate cerebral edema after ICH (Wu *et al*., 2008). The direct administration of r-hirudin to hematoma cavern inhibited the up-regulation of AQP-4 and AQP-9 induced by thrombin and significantly decreased the expression of both AQPs (Sun *et al*., 2009), suggesting that reducing the effect of thrombin and AQP-4 using an inhibitor or an antagonist might help reduce brain edema after ICH. However, the time window for administering a thrombin inhibitor or antagonist is poorly understood.

The present study was designed to measure the mRNA and its protein expression levels of AQP-4 and the BWC in perihematomal brain tissues in a rat model of ICH. Then, the thrombin inhibitor hirudin was injected into the hematoma at different time points after the successful induction of ICH to determine the optimal time window for hirudin administration.

MATERIALS AND METHODS

Reagents and drugs: The primary reagents and drugs used in this study were as follows: hirudin (Tianjin Taide Biological Products Co., LTD, China), Trizol (Invitrogen Corporation, USA), phenol/chloroform(Shanghai Shenggong Biological Technology Co., LTD, China), Superscript II retrovirus kit (Invitrogen Corporation, USA), PCR kit (Promega, USA), BCA method protein concentration determination kit (Pierce Company, USA), rabbit anti-human AQP4 polyclonal antibody(Santa Cruz Biotechnology, USA), protein molecular markers(Amersham Bioscience Company, USA), tetramethylethylenediamine(TEMED, Sigma Company, USA), ammonium persulfate (APS, Sigma Company, USA), three hydroxyl methyl carbamate methane (Tris, Shanghai Biological Chemical Company, China), ECL protein luminescence kit (Amersham Bioscience Company, USA), PVDF membrane (Millipore Company, USA), PCR primer synthesis (Shanghai Shenggong Biological Technology Co., LTD, China), DNA Marker DL2000 (D501A, Takara Company, Japan) and Taq DNA polymerase (Invitrogen Company, USA).

Main instruments: The following instruments were used in this study: stereotaxic frame (DYI-I, Tianjin, China), electronic balance(Sartorius AG, Germany), low-temperature desktop centrifuge(Heraeus Company, USA), mini-gel electrophoresis spacers(H6-1, Ningbo Instrumentation Institute, China), dimension quantity infusion pump (Medical Instrument Plant of Zhejiang College, China), PCR thermal cycler (PerkinElmer, USA), UV spectrophotometer (DU530, Beckman, USA), electronic balance (FA1604S, Shanghai Balance Instrument Plant, China), -80°C freezers(Forman Scientific, USA), CS20 liquid nitrogen containers(MVE, Inc.), vertical electrophoresis apparatus and electrophoretic transfer cell (Bio-Rad Company, USA), gel imaging system (Gel Doc 2000, Bio-Rad Company, USA), trace pipette(Eppendorf Company, Germany), protein electrophoresis apparatus (Bio-Rad Company, USA), microscopic camera (Polaroid, USA), BX51 optical microscope (Olympus, Japan) and microscopic image analysis system (Zeiss Company, Germany).

Experimental groups: The protocols of the study were approved by The Animal Care and Use Committee of Guiyang Medical University.

Forty-eight healthy male Wistar rats weighing from 350-450 g were used in this study. The 48 animals were randomized to the control group (MC group, 12 rats) or the medical treatment group (MT group, 36 rats). The latter group was equally subdivided into 6, 12 and 18 h MT groups, with each group including 12 rats.

ICH model induction: The animals were anesthetized using 10% chloral hydrate (0.035 mL kg^{-1}). The rats were positioned in a stereotaxic frame (type DYI-I, Tianjin, China) and a cranial burr hole (1.0 mm) was drilled in the skull (0.2 mm anterior, 5.5 mm ventral, 3.0 mm lateral to Bregma). Approximately 50 μL autologous whole blood was collected from the femoral artery of each rat using a sterile syringe. A 25 gauge needle was attached, the

syringe was secured in the frame and the needle was quickly introduced into the caudate nucleus. The depth of the caudate nuclei is approximately 6 mm from the skull surface. The blood was injected into the rat brain at a rate of 5 μL min^{-1} using a controller affixed to the stereotaxic frame. The total time of injection was approximately 10 min. The needle was left in place for 5 min and then removed slowly.

Hirudin intervention: Hirudin (4 μL solution, containing 15 units of hirudin) was injected directly into the hematoma cavern in the 6, 12 and 18 h MT groups. In the MC group, however, only 0.9% sodium chloride solution in the same amount was injected.

Sample collection: All of the animals were sacrificed 48 h after the successful induction of ICH. The chest of each animal was opened to perfuse the heart using a 0.9% sodium chloride solution, followed by removal of the brain. The needle track was used as the site to prepare coronal and sagittal sections, so the brain on the hematoma side was sliced and divided into four parts. The brain tissues collected from the front-inner region was used to measure the protein expression of AQP-4 via Western blot, the front-outer region was used to measure the mRNA expression of AQP-4 via RT-PCR and the other regions were used to measure the BWC.

BWC determination: The dry and wet weight method was performed as described previously (Lin *et al*., 1993) to measure the BWC. The BWC was calculated as (wet weight-dry weight) /wet weight×100% (Yang *et al*., 1994).

Determination of AQP-4 mRNA expression via RT-PCR: Brain samples (approximately 50 mg) extracted from the front-outer region of the hematoma were pulverized (Xi *et al*., 1999) and total RNA was isolated using TRIzol reagent. cDNAs were generated from 8 μL (0.5 μg μL^{-1}) of the total RNA using Superscript II RNase H-reverse transcriptase (RT) primed with 1 μL (0.5 μg μL^{-1}) oligo (dT). The primers for AQP4, β-actin and the PCR protocol were designed as follows: AQP4: 5'-GTCTTTCCCCTTCTTCTCCTCTCC-3' (forward), 5'TGCATTCACATTTGTTGTGC-3' (reverse); β-actin: 5'-GCCAGCTCACCATGGATGAT-3' (forward), 5'-AGAAGGTGT GGTGCCAGAT-3' (reverse). PCR of multiple targets was performed by co-amplifying β-actin as an internal standard. The reaction mixture for detection of AQP4 mRNA contained 9 μL of the RT reaction product and the reaction protocol was 1 cycle for 2 min at 94°C, 35 cycles of 45 s at 94°C, 1 min at 62°C and 1 min at 72°C, followed by 10 min at 72°C. The specificity was verified via electrophoresis of the PCR products, which displayed only one band on the gel for each PCR.

Western blot for AQP-4 protein: Brain tissues were ground in liquid nitrogen. Sample buffer (200 μL, 1×SDS) was added to 100 μg brain tissues; then, the tissues were blended. The samples were centrifuged for 30 min at a temperature of 4°C. The supernatant was removed and the total protein was extracted. For Western blot for AQP4, the tissues were dissolved in 20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 2 mM EDTA, 1% Na_3VO_4, 10 lM leupeptin, 10 lM pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF) on ice in Eppendorf tubes. The protein samples (50 μg) were loaded on a 12% SDS-polyacrylamide gel for electrophoresis and were then transferred to nitrocellulose membranes at 0.8 mA cm^{-2} for 2 h. The membranes were blocked at room temperature for 1 h using blocking solution (5% skim milk in Tris-buffered solution containing tween 20 (TBST): 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% v/v Tween 20). The membranes were then incubated overnight at 4°C in the anti-AQP4 rabbit polyclonal antibody diluted 1:200 in the blocking solution. After two 10 min washes with TBST, the membranes were incubated for 1 h at room temperature in Alexa Fluor 800 or Alexa Fluor 700 conjugated goat anti-rabbit secondary antibody diluted 1:2000 in blocking solution. The Gel Doc 2000 gel imaging system and software (Bio-Rad) were used for imaging and quantification using grayscale analysis. Semi-quantitative analysis was performed using GAPDH as an internal reference.

Statistical analysis: The statistical analysis was performed using the SPSS 11.5 software. The Kruskal-Wallis test (H statistic) was used to compare the differences in the BWC and the AQP-4 mRNA expression levels and a one-way analysis of variance was used to compare the differences in the AQP-4 protein expression levels. When a significant difference was detected, Scheffe *Post-hoc* analysis was performed to identify the significant group differences. All of the results are presented as the Means±SD, $p<0.05$ indicated a significant difference. The statistical analysis was performed in consultation with the Health Statistics Department of Guiyang Medical University.

RESULTS

BWC measurement: The Kruskal-Wallis Test was used to compare the differences between the groups, as the BWC data did not meet the criteria for analysis of variance. A significant difference was detected between

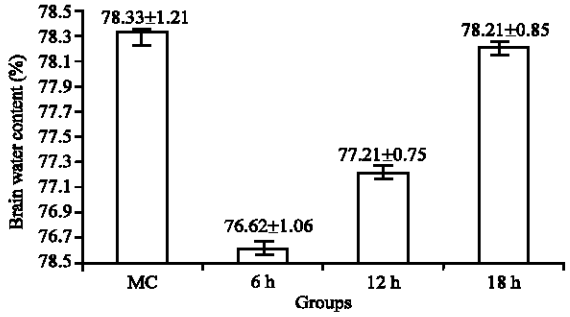

Fig. 1: Effects of hirudin administered at different time points on the BWC. The BWC decreased significantly after the administration of hirudin compared to the MC group. The effectiveness of hirudin to decreasing the BWC varied depending on the time point of hirudin administration. The BWC in the 6 h group was the lowest

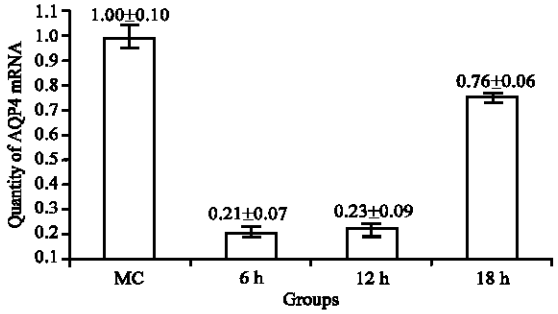

Fig. 2: Effects of hirudin administered at different time points on the expression of AQP-4 mRNA. The expression level of AQP-4 mRNA significantly decreased after hirudin treatment, especially in the 6 and 12 h group. As the time point of administration prolonged, the effectiveness of hirudin in decreasing the AQP-4 mRNA expression level was reduced

the groups (χ^2 = 15.525, p = 0.001). Further analysis revealed that the difference between every two groups was statistically significant. The BWC in each MT group was significantly decreased (especially the 6 and 12 h groups) compared to the MC group, suggesting that the direct administration of hirudin to the hematoma in the early stage (6-12 h) remarkably decreased the perihematomal BWC, thereby reducing cerebral edema. Hirudin administered 18 h after hemorrhage onset also decreased brain edema formation (Fig. 1) but the effect was significantly less than that of the 6 and 12 h groups.

Expression of AQP-4 in the perihematomal brain tissue: A statistical analysis of AQP-4 mRNA expression

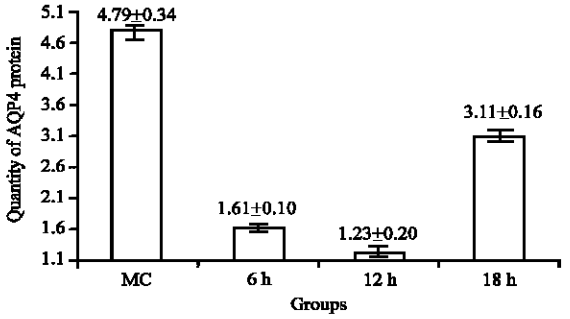

Fig. 3: Effects of hirudin administered at different time points on the expression of AQP-4 protein. The quantity of AQP4 protein after administration of hirudin significantly decreased compared to the control, especially in the 18 h group, suggesting that administering hirudin at an early stage could significantly inhibit AQP-4 protein expression. As the time window prolonged, the effectiveness of hirudin in decreasing the AQP-4 protein expression level was reduced

was also performed using the Kruskal Wallis Test. A significant difference in the mRNA expression of AQP-4 was detected between the groups (χ^2 = 14.932, p = 0.002). Further analysis using a Scheffe post-hoc analysis revealed that the difference between every two groups was statistically significant, except the difference between the 6 and 12 h groups. The mRNA expression levels of AQP-4 in the 6 and 12 h groups were significantly decreased compared to the control group. A significant difference in the relative quantity of AQP-4 protein between the groups was also found (one-way analysis of variance, F = 5827.46, p = 0.000). Further analysis using a Scheffe post-hoc analysis revealed that a significant difference existed between every two groups. The relative quantity of AQP-4 protein was the lowest in the 12 h group. These results suggested that the administration of hirudin at early stages reduced the mRNA and protein expression levels of AQP-4 ($p<0.05$; Fig. 2 and 3, respectively). The mRNA and protein expression levels of AQP-4 in the 18 h group displayed a small decrease compared to the control but a significant increase compared to the 6 and 12 h groups ($p<0.05$), suggesting that the administration of hirudin 18 h after ICH onset has a minor reducing effect on AQP-4 mRNA and protein expression.

DISCUSSION

Brain edema formation is the most frequent complication after ICH, which leads to the deterioration of neural function and death, but the mechanism by which brain edema occurs remains unknown. Recent

studies have demonstrated that AQP-4 plays an important role in the formation of brain edema after ICH (Lee *et al*., 1997; Bartha *et al*., 2000; Matsuoka and Hamada, 2002; Lu and Sun, 2003). ICH significantly increased the BWC, the permeability of the BBB and the expression level of AQP-4 mRNA. Both the mRNA and protein expression levels of AQP-4 positively correlated with the permeability of the BBB (Dai *et al*., 2006).

In the present study, the perihematomal BWC increased and the mRNA and protein expression of AQP-4 was up-regulated after the successful induction of ICH. Hirudin injected directly into the hematoma at different stages after the induction of ICH decreased the perihematomal BWC, leading to a reduced severity of brain edema. Simultaneously, the mRNA and protein expression levels of AQP-4 were decreased compared to the MC group. The present results suggested that thrombin-induced brain edema might be caused by the up-regulation of AQP-4. An inhibitor of thrombin (hirudin) down-regulated the mRNA and protein expression of AQP-4, thus reducing cerebral edema. These results were consistent with previously published studies (Sun *et al*., 2009). Thrombin, which is produced during the blood coagulation process, plays a pivotal role in the formation of brain edema (Lee *et al*., 1996, 1997; Matsuoka and Hamada, 2002; Xi *et al*., 2003, 2006; Hua *et al*., 2007). The administration of a thrombin inhibitor effectively limited brain edema formation and neuronal damage, thereby improving survival and functional outcome; antithrombin therapy via intravenously administered argatroban may thus be useful for the treatment of ICH (Matsuoka and Hamada, 2002). The expression levels of several proteins, including AQP-4 and AQP-9, were altered due to thrombin infusion, which may be related to thrombin-induced acute brain edema after ICH; therefore, reducing the effect of thrombin using hirudin could inhibit the up-regulation of AQP-4 and the increase in the BWC (Sun *et al*., 2009). However, the time window for administering hirudin to reduce the BWC and the expression levels of AQP-4 remained poorly understood. AQP-4 mRNA expression began to increase at 6 h after blood infusion and peaked at 72 h, while AQP-4 protein expression began to increase at 24-48 h and peaked at 72 h (Dai *et al*., 2006; Sun *et al*., 2009). If the thrombin-induced up-regulation of AQP-4 mRNA expression was blocked or inhibited at the early stages preceding the increase in APQ-4 protein expression, the BWC should be reduced. This simple hypothesis was demonstrated in the present study. In our study, a rat model of ICH was used as the experimental model and the effect of hirudin administered at different stages after the successful induction of ICH on the perihematomal BWC and the expression of AQP-4 was measured. The results revealed that the administration of hirudin at early stages (6-12 h) significantly down-regulated the mRNA and protein expression levels of AQP-4 and decreased the BWC. The administration of hirudin 18 h after ICH induction, however, had only a small reducing effect on the AQP-4 expression levels and the BWC. These results suggested that the optimal time window for administering hirudin to prevent brain edema formation might be within 12 h of hemorrhage onset.

The aim of this study was to provide another option for the treatment of secondary brain damage after ICH. Previously published studies have demonstrated that a range of factors, including thrombin and AQP-4 were involved in the development of BBB disruption and brain edema. Therefore, blocking the effects of thrombin and AQP-4 or reducing their production could be of some value for minimizing brain damage. Hirudin was administered directly into the hematoma using a surgical procedure in this experiment. Clinically, such surgical procedures are typically performed to evacuate the hematoma. However, brain damage remains serious after evacuating the hematoma, even if the procedure is performed using a MIS (Wu *et al*., 2011), as a hematoma exists for some time until it is evacuated via a surgical procedure. Furthermore, the minimally invasive procedure is to stereotactically insert a tube into the hematoma and initially remove a large part of the hematoma, followed by infusing fibrinolytic agents to dissolve the clots (Barrett *et al*., 2005; Miller *et al*., 2007; Morgan *et al*., 2008; Wang *et al*., 2009). Thus, the residual hematoma continues to be present and the brain damage continues. Therefore, a minimally invasive surgery followed by the administration of drugs to prevent brain edema would be beneficial for minimizing brain damage. The present study was based on the effects of thrombin and AQP-4 on the formation of brain edema and the administration of hirudin inhibited the up-regulation of AQP-4 expression and the development of brain edema, as described in the introduction. Then, we focused on the optimal time window of hirudin administration on AQP-4 expression without determining the range of brain edema or its type, which represents a limitation of the present study. Further experiments are required to demonstrate the effective time window of hirudin administration on AQP-4 expression and brain edema via histological identification of the presence and type of brain edema.

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