

Original Research

Expression and significance of aquaporin protein in sprague-dawley rats after experimental intraventricular hemorrhage

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Abstract: The AQP4 protein in Sprague-Dawley (SD) rats was expressed after experimental intraventricular hemorrhage to investigate the possible pathogenesis of chronic posthemorrhagic hydrocephalus. Seventy SD rats were randomly divided into two groups – sham-operated group (n=35) and experimental group (n=35). Saline and citrated autologous blood were injected into the lateral ventricle of the SD rats in the two groups, respectively. The rats in experimental group were further divided into seven subgroups based on different time points at 3 h, 6 h, 12 h, 1 d, 3 d, 5 d and 7 d. The change of expression of AQP4 protein at different time of bleeding were detected by immunohistochemical techniques and mRNA of AQP4 was observed by in situ hybridization. It was found that 75% rats in experimental group suffered from chronic hydrocephalus at 12 h after intraventricular hemorrhage. High protein expression of AQP4 was observed in the apical of cuboidal epithelium of choroids plexus. The protein expression of AQP4 gradually weakened in experimental group after 3 days of intraventricular hemorrhage and dropped to the minimum at the 7th day, which was significantly different from the control and operative groups ($P<0.05$). The mRNA expression of AQP4 was weaker than the protein expression, and the locations of expression were generally in agreement with each other.

Key words: Intraventricular hemorrhage, posthemorrhagic hydrocephalus, immunohistochemical, Aquaporin, gene expression.

Introduction

An intraventricular hemorrhage is a bleeding into the brain's ventricular system, where the cerebrospinal fluid is produced and circulates through towards the subarachnoid space (1-3). It can result from physical trauma or from hemorrhaging in stroke (1-5). So far, the mechanisms of chronic hydrocephalus after cerebral hemorrhage is still unclear, most of cases have been considered to the connection of the subarachnoid particles and fiber hyperplasia, resulting in malabsorption of the cerebrospinal fluid circulation and finally lead to hydrocephalus.

Aquaporins are integral membrane proteins from a larger family of major intrinsic proteins that form pores in the membrane of biological cells (6-13). Aquaporins selectively conduct water molecules in and out of the cell, while preventing the passage of ions and other solutes, which are also known as water channels, aquaporins are integral membrane pore proteins, some of them were known as aquaglyceroporins, which also transport other small uncharged solutes, such as glycerol, CO_2 , ammonia and urea across the membrane, depending on the size of the pore (6,8,9,11-13). The AQP1 channel has a pore width of 8-10 Angstroms and allows the passage of hydrophilic molecules ranging between 150-200 Da (7,8). However, the water pores are completely impermeable to charged species, such as protons, a property critical for the conservation of the membrane's electrochemical potential difference. The main functions of AQP4 have been considered to involve into the secretion of cerebrospinal fluid, and to maintain the osmotic pressure and the balance between water and electrolyte (9-12). However, there have been no reports about whether AQP4 protein is involved in the absorption of cerebrospinal fluid and formation of chronic hydrocephalus. In order to learn more about the mechanisms of chronic

hydrocephalus after cerebral ventricular hemorrhage, in this study the expression of AQP4 protein and AQP4 mRNA in the brain of SD rats after experimental cerebral hemorrhage was observed and discussed to explore the relationship between the expression of protein and chronic hydrocephalus.

Materials and Methods

Experimental animals

Seventy SPF grade SD rats (age: 8-10 weeks, weight: 350-400 g) were purchased from Animal experimental center of Fundamental Medical College at Handan Medical University. Temperature and humidity were controlled at 25 ± 1 °C and 55%-60%, respectively. There were no restrictions on drinking water and feeding. The rats were divided into the sham-operated group and experimental group with 35 rats in each group. The rats in experimental group were further divided into seven subgroups (5 rats in each subgroup) according to experimental time points at 3 h, 6 h, 12 h, 1 d, 3 d, 5 d and 7 d.

Reagents

RT-PCR reagent kits (Instant PCR Kit 3.0) and total DNA extraction reagent (Tiandz RNAiso Reagent) were purchased from Beijing Tiandz, Inc. PCR maker was provided by Tianjin Jiangmei Company (100-1500 bp).

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Up primer and down primer of PCR were synthesized by Shanghai Tongwei Reagent Company.

Instruments

High precision electronic balance machine (Beijing Kerui); high speed centrifuge (GTR16-2, Beijing Era Beili Centrifuge); UV-vis spectrophotometer (Angilent Cary-300); PCR amplification instrument (Thermo Scientific Piko); electrophoresis tank (Bioduro tech); gel automatic imaging instrument (Gel-Pro_analyzer 4.0).

Model establishment

The animal model and water content in brain tissue were established according to a published method (14).

AQP4 detection

Immunohistochemical method (ABC method) was used, the details are described as follows. Thick frozen sections (6 μm) were placed at room temperature for 40 min and fixed in cold acetone for 20 min, H_2O_2 (3%) was used to inactivate endogenous peroxidase for 10 min, followed by dripping with normal goat serum and sealed for 40 min, then placed for overnight at 5 °C. The sample was washed with 0.01% PBS, biological prime coupled second antibody (anti rabbit IgG) was added by droplet, and incubation for 40 min at 37 °C, oxidase conjugated streptavidin egg white was added by droplet, and incubation for 40 min at 37 °C. After hematoxylin staining for 10 min, the sample was sealed for observation of AQP4 proteins.

Statistical methods

Statistical analysis of the expression of AQP4 and brain water content is expressed by $\bar{x} \pm s$. Statistical method is described as follows. First, database is constructed for the normality test of data, the following test was conducted under the condition of normality; second, each model of the operated side and non operated side were compared using the paired t test; third, comparison was made between the brain hemorrhage control group, experimental group and sham-operated group for the overall difference, single factor variance analysis was used, if overall difference was found, SLD method was applied for model pairwise comparison between each of the two groups; fourth, the single factor variance analysis was used for cerebral hemorrhage comparison of the overall difference between the groups, if overall difference was found ($P < 0.05$), SLD method was applied for model pairwise comparison between each of the two groups; fifth, the analysis between AQP4 expression of surgical or non-surgical side and brain water content was conducted using SPSS 13.0 statistical software, $P < 0.05$ shows that the difference exists, $P > 0.05$ indicates that the difference does not exist.

Results

HE staining

After 6 h of intracerebral hemorrhage, the red blood cells in hematomas kept their initial profiles, a small amount of individuals can be locally observed, the cells scattered or infiltrated in spherical or oval nucleus of

trachychromatic inflammatory cells. After 24 h of operation, infiltration of inflammatory cell was mainly mononuclear cells, and a few neutrophils were observed. After 48 h of operation, infiltration of inflammatory cells was mainly neutral points granulocyte with clumped distribution. After 72 h of operation, the number of inflammatory cells decreased, proliferation of gliocytes around the hematoma and inside hematoma was observed. After 7 days of operation, the hematoma was significantly reduced, the gliocytes were clearly observed. There were no hematoma and significant inflammatory cell infiltration observed in the samples taken from the sham-operated group.

AQP4 expression

The scattered AQP4 cells (mostly gliocytes) are visible in the normal group, granular deposition in cytoplasm and nucleus can be observed in Figure 1. The number of AQP4 cells increased after 1 h of operation in experimental group, and reached the peak at 48 h after operation, the peak continued for 7 days. The expression of AQP4 was mainly gliocytes (see Figure 2), and there is no significant difference between the expression of the hematoma side and AQP4 of contralateral brain tissue (see Table 1). A small amount of expression was also found in the distant parts of cerebellar hemorrhage.

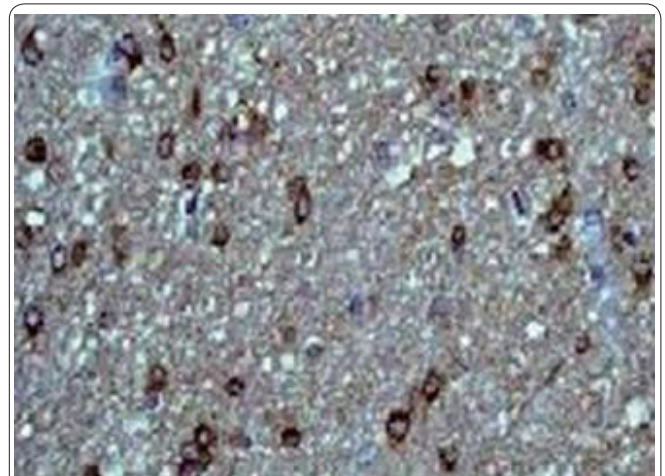


Figure 1. Expression of AQP4 in brain tissue of sham-operated group, 400x.

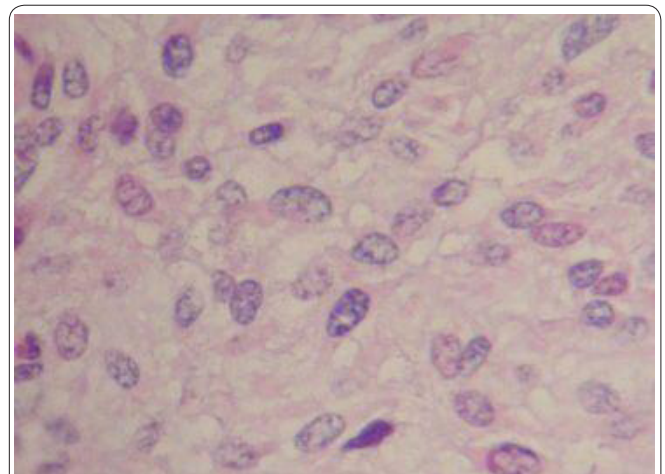


Figure 2. Expression of AQP4 in brain tissue after 24 h of intraventricular hemorrhage, 400x.

Table 1. The number of AQP4 cells in the lateral and contralateral cerebral tissue of intracerebral hemorrhage (expressed by $\bar{x} \pm s$).

hemorrhagic time	lateral cerebral tissue	contralateral cerebral tissue
3 h	4.13±1.27	3.84±1.55
6 h	6.42±1.58	5.28±1.37
12 h	8.64±1.75	7.04±1.46
1 d	24.86±3.51	22.77±2.06
3 d	32.39±3.49	31.73±3.19
5 d	18.22±2.37	17.65±1.51
7 d	4.97±1.53	5.35±1.16
sham-operated group	3.48±1.39	2.82±0.97

The change of cerebral edema after cerebral hemorrhage

From 24 h after cerebral hemorrhage, the basal ganglia region and the corresponding cortical area start to form obvious edema, the edema peak appeared at 48 h, and the peak continues for 7 days. The water content of the brain tissue was also higher than that of the sham-operated group at the opposite side of the hematoma.

Discussion

Cerebral edema is a very important complication of cerebral hemorrhage, moreover it is an important reason for disease aggravation and death in patients with cerebral hemorrhage (15,16). Some studies indicated that appearance of cerebral edema after hemorrhage might be related to the following factors: 1. Some harmful substances in hematoma fluid, such as red blood cells, hemoglobin, white blood cell, thrombin and complement; 2. The functions of some enzymes, such as heme oxygenase (HO); 3. Some inflammatory cytokines such as TGF- α , IL-6, IDEM-1, etc. The concomitant inflammatory reaction after cerebral hemorrhage has been confirmed, the infiltration of a large number of neutrophils can be observed in the model of rat cerebral hemorrhage induced by collagenase (17,18). The infiltration started between 6 and 12 h, and reached peak between 1 and 3 d. Neutrophils are capable of releasing potentially harmful substances such as oxygen free radicals or cytokines, such as TGF- α , IL-6, and ITG- γ , which are significant in cerebral injury. Neutrophils can also result in local ischemia by occlusion of capillaries to cause edema of the cerebral tissue. The reported peak times of cerebral edema after hemorrhage were different in previous studies. However, it was generally considered to be 1 to 3 d after hemorrhage. Therefore, the peak time of infiltration of neutrophils and cerebral edema after hemorrhage are consistent with each other. AQP4 protein can be considered as a regulatory protein with pleiotropic transcription, which exists widely inside various cells, such as T lymphocytes, monocytes/macrophages, polymorphonuclear cells, vascular endothelial cells, epidermal cells, fiber cells, neuronal cells, astrocytes and so on. AQP4 protein is a trimer composed of three protein subunits including P55, p60 and p65, at resting state AQP4 associated with its profilin I-AQP4 to form a tetramer, which present in the cytoplasm with an inactive form. When certain stimuli are subjected, under the presence of specific protein kinase, I-AQP4 degrades after phosphorylation, AQP4 can be released from the tetramer in cytoplasm, and rapidly enter the

nucleus to regulate a series of gene expression. Genes of encoding inflammatory molecules are responsive genes of AQP4. The primary function of AQP4 is transcription and regulation of inflammatory molecules such as TGF- α , IL-3, 7, 8 and IL-9, transfer growth factor and intercellular adhesion molecule-1 (ICAM-1), iNOS, cyclooxygenase-2 and glial fibrillary acidic protein (GFAP).

In this study, it was found that after cerebral hemorrhage, the number of AQP4 cells began to increase at 3 h after operation, reached peak at 1 d, and continued for 3 days. The expression is mainly glial cell. There was no significant difference between the expression of the hematoma side and the contralateral AQP4 in cerebellar hemorrhage. Cerebellum also expressed a small amount of AQP4 in distant parts. The sham-operated group had only a small amount of AQP4 cells. Cerebral edema started at 12 h after intracerebral hemorrhage, reached peak at 48 h, and continued for 7 days. Inflammatory cell infiltration appeared at 12 h after cerebral hemorrhage, reached peak at 48 h up, and continued for 7 days as well, suggesting that there is a correlation between the peak of cerebral edema and inflammatory cell infiltration and the expression peak of AQP4. As a result, it can be proposed that AQP4 might be another reason for cerebral edema after hemorrhage. Under the external stimulation, glial cells and neurons of AQP4 gene are able to encode TGF- α , IL-6 and other inflammatory factors, TGF- α can further activate the AQP4, consequently the inflammatory reaction is enlarged. Roseberg *et al* (19) believed that there is a significant correlation between TGF- α and hematoma around cerebral edema, TGF- α can be considered as a signal of cerebral edema caused by inflammation resulting from hematoma. Auphen *et al* (20) has found that intracerebroventricular injection of TGF- α can increase capillary permeability, the mechanism is destruction of membrane in capillary basement due to combination of TGF- α and gelatinase B. Therefore, TGF- α can damage barrier between blood and cerebrum and cause cerebral edema. This study also found that AQP4 was expressed in the cerebral cortex, hippocampus and cerebellum synapses, AQP4 exist at the presynaptic terminal, the postsynaptic region and inside the nucleus, local synaptic signal will induce its activation. The active AQP4 can be transported into the nucleus along the opposite direction from any parts of the axon, which acts as a signal to link the synapse and nuclear events, it simultaneously plays a biological role in the regulation of gene transcription in the nucleus. Therefore, it can be proposed that the bilateral AQP4 expression does not have significant difference, the

cerebellum in the distant area of hemorrhage also has expression of AQP4, which is probably related to the effective activation of AQP4 by electrical activity inside neurons and synaptic transmission between neurons. The water content of the hematoma side was higher than that of the contralateral cerebral tissue, which may be related to comprehensive factors as the toxic effects of hematoma and the activation of AQP4. In contralateral cerebral tissue, the brain water content was also higher than that of sham-operated group probably related to the activation of AQP4 in contralateral cerebral tissue.

In summary, AQP4 might be an important factor to cause cerebral edema after hemorrhage. Investigation on the mechanisms of AQP4 can provide a new therapeutic strategy for the treatment of cerebral edema after hemorrhage.

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