Original Article
Aquaporin-4 expression in developing rat brain following status epilepticus (SE) and the effects of exogenous GM1 on it

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Abstract: Objectives: The aim of study was to investigate the relationship between aquaporin-4 (AQP4) and brain edema following status epilepticus (SE) via observing the changes of AQP4 expression in brain edema formation following SE in the developing rats, We also observed the effects of Monosialote-trahexosylganglioside (GM1) on the expression of AQP4 and brain edema following SE, contributing to learn more about the protective function of GM1 on brain edema due to SE. Methods: One hundred and fifty male developing Spraque-Dawley (SD) rats were randomly divided into three groups: control group, status epilepticus group (SE group) which was induced by pilocarpine (PILO), and GM1 group (SE+GM1), in which the rats were given intraperitoneal injection of GM1 30 mg/kg after SE, once again every 24 h for six times. Each group was subjected to 4 subgroups: 6 h, 24 h, 72 h and 7 d after SE (n=5). Each brain tissue was respectively used to observe the changes of morphology, to determine the brain water content (BWC) by brain wet-to-dry weight ratio and to determine the expression of AQP4 by using immunohistochemical staining and to be semi quantified as an optical density (OD) value of positive cells with pathology imaging analysis system (PIAS). Results:In control group, there were no significant changes of the morphology, both the brain water content and the expression of AQP4. In SE group, HE staining showed swelling, degeneration of the brain cell and proliferation of astrocyte, so did the changes of SE+GM1 group. Compared with control, SE group significantly increased BWC and the expression of AQP4, all began at 6 h and peaked at 72 h ($P<0.05$), but no variance at 7 d ($P>0.05$). AQP4 expression positively correlated with BWC (r=0.623, $P<0.01$). In SE+GM1 group, compared with SE group, the elevation of BWC was strongly attenuated at all time point except 7 d. While AQP4 levels demonstrated an obviously atenuation from 24 h. At 72 h, the AQP4 OD value decreased from 0.396±0.026 (SE group) to 0.243±0.036 (SE+GM1 group_) ($P<0.05$). Conclusions: Cerebralcortical AQP4 expression is up-regulated after SE with the deterioration of brain edema. It reveals that AQP4 participates in the development of brain edema after SE. Exogenous GM1 may inhibit AQP4 expression after SE and attenuate brain edema.

Keywords: Status epilepticus, brain edema, aquaporin-4, GM1

Introduction

Status epilepticus (SE) is a common clinical critical illness. Since brain edema (BE) is an important cause, leading to the lethality and dysfunction of the acute phrase of SE, prevention and elimination of BE is the key to the treatment of SE [1]. Currently, the treatment of cerebral edema is limited to hypertonic dehydration and surgical decompression. There is no effective treatment against the molecular mechanism of cerebral edema. The treatment of SE is often unsatisfactory, and cannot reach the purpose of the prevention of cerebral ed-

ema. Causes of brain edema formation are multifactorial. It is conventionally thought that the depletion of energy and calcium overload [1]. However, the conventional through cannot give an entire and reasonable interpretation to water transportation and regulatory mechanisms.

Recent studies have shown that aquaporin-4 (AQP4) is an important molecule involved in the formation of BE and plays an important role in fast transmembrane water transport. Foreign data [2-7] have proven that AQP4 participates in the formation of BE after central nervous sys-

tem injury. Studies by Manley et al. [8] on the pathogenesis of BE caused by AQP4 in the rat acute water intoxication have shown that AQP4 expression increase can be detected in the rat acute water intoxication. In addition, Manley et al. [8] found that brain water contents decreased and that edema in the astrocytes significantly reduced in the BE model in which AQP4 expression was down-regulated, suggesting that AQP4 is involved in the formation of BE. AQP4 also play an important role in the pathogenesis of BE after ischemic, hypoxic, traumatic and hemorrhagic brain injury. For example, researchers [9] found that AQP4 mRNA and protein expressions up-regulated 1 day after artery occlusion in the model of ischemic BE, increased significantly on day 3 and decreased on day 7, which was in parallel with the formation and development of ischemic BE. The results supported the hypothesis that AQP4 is involved in the ischemic BE. Currently, there are relatively few reports on AQP4 expressions after status epilepticus (SE) in China and abroad. Therefore, studies on changes in AQP4 expressions in the formation of BE after SE will contribute to the treatment of BE.

Monosialote-trahexosylganglioside (GM1), also known as single four hexose ganglioside sialic acid, is a neuroprotective agent, which the brain is rich in. GM1 has broad neuroprotective effects. When the central nervous system is damaged, exogenous GM1 can easily pass through the blood-brain barrier, and embed into the surface of the cell membrane, playing functions similiar to some functions of endogenous gangliosides and thus exerting neuroprotective effects [10]. Research has confirmed that GM1 has definite protective effects on brains after SE, cerebral ischemia, cerebral hypoxia and brain injury [11]. However, the recent research on the effects of GM1 on AQP4 expressions and how GM1 mitigate BE is relatively little.

This experiment adopted the SE model of developing rats in which SE was induced by the intraperitoneal injection of pilocarpine (PILO). The relationship between AQP4 and BE following SE was studied through observation of changes in AQP4 expressions and brain water contents in the developing rats after SE at different time points in order to explore the mechanism of BE formation after SE. Meanwhile, the effects of GM1 on AQP4 expressions and BE after SE

were observed to investigate the mechanism of protective effects of GM1 on BE after SE, providing new methods for the clinical prevention and treatment of BE.

Materials and methods

Experimental animals

Healthy Sprague-Dawley (SD) rats (weighed 50-55 g, aged 21 days) were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences, and randomly divided into three groups: a control group, status epilepticus group (SE group) and GM1 group (SE+GM1). Each group was categorized into 4 subgroups: 6 h, 24 h, 72 h and 7 d after SE (n=5). Brain water contents and AQP4 protein in the brain were detected respectively.

Model establishment [12] and sample preparation

Developing SD rats were given a peritoneal injection of methyl scopolamine (1.0 mg/kg i.p.) to reduce peripheral cholinomimetic side effects of PILO. Rats were given an intraperitoneal injection of PILO (180-225 mg/kg) 30 minutes later. Evaluation standard of successful modeling: seizure activities were divided into VI levels according to 6 levels set by Racine [13]. Level 0 was given when there was no evidence of seizures; level I was given when gazing, chewing and moving of lips were observed; level II was given when nodding and wet dog shakes were observed; level III was given when limited forelimb clonus occurred; level IV was given when generalized tonic-clonic seizures accompanied with standing of hind limbs occurred; level V was given when generalized tonic-clonic seizures accompanied with standing and falling happened. Among rats which were graded as level IV or V, rats in which generalized tonic-clonic SE lasted for 1 h were included in the model of SE. Rats which did not reach the above standard or died were discarded. When seizures continued for 1 h and were still not alleviated, intraperitoneal injection of chloral hydrate (400 mg/kg) was given to terminate seizures. The time point when SE was terminated was used as the start time point to calculate time. The control group was injected with the same volume of saline. After successful modeling, rats were randomly divided into groups SE and SE+GM1 groups with 20 rats in

each group. These rats were immediately given an intraperitoneal injection of GM (130 mg/kg), and given an intraperitoneal injection of GM again 24 later. Then the intraperitoneal injection of GM continued for 6 days. Meanwhile, rats in the control group and SE group were injected with the same volume of normal saline. The experimental drug was monosialotetrahexosylganglioside (GM1) aqueous solution (trade name Shenjie).

Methods

Determination of brain water contents: Dry and wet weights were measured. Animals were sacrificed at a predetermined time point. Brains were quickly removed. After filter paper absorbed all blood on the brain surface, left hemisphere of brain tissue was removed. After the wet weight of left hemisphere was measured, tissue blocks were dried in an oven at a constant temperature (100°C) for 24 h. After the dry weight was measured, the Elliot formula was applied to calculate percentages of brain water contents at each time point. Brain water content (%) = (wet weight-dry weight)/wet weight ×100%.

Material used in the experiment: Pilocarpine purchased from Sigma Company. Methyl scopolamine purchased from Sigma Company. GM1 (trade name Shenjie) purchased from Qilu Pharmaceutical Company. Primary antibody: monoclonal antibodies rat anti mouse AQP4 purchased from Wuhan Boster Biotechnology Company. Secondary antibody: biotinylated secondary antibodies. SABC immunohistochemistry kits purchased from Wuhan Boster Biotechnology Company. Antibody dilution purchased from Beijing Zhongshan Biotechnology Co., Ltd. APES purchased from Sigma Company. DAB color reagents (Shanghai Zeheng Biotech Company, Lot: 50281313).

Main instruments: Paraffin slicing machine (MICROW HM34OE Germany). Fluorescence microscope camera: OLYMPUS BX60, Japan. Embedding machine and freezing machine (BMJ-III-type, Jiangsu, China). Water jacket incubator (GNP-9080, Shanghai, China). HPIAS-1000 pathological image analysis system (Wuhan Tongjiqianping Screen Image Engineering Company, China). DHG-9140A electric oven thermostat blast. Electronic balance (Shanghai Experimental Instrument Factory).

Experiment

Animal grouping: 150 health male SD rats aged 21 days.

Preparation of animal models [12].

Preparation of tissue sections: The right sides of the rat brain tissues were fixed with 10% neutral buffered formalin (PH 7.4) for 8-12 hours; Washed with water for 30 min; 70% ethanol 30 min; 95% ethanol 1 h ×2 times; 100% ethanol 1 h ×2 times; Transparent by xylene 20 min ×2 times; Dipped in wax 58-60°C 3 h; Embedded; After embedded in paraffin, tissues were sliced consecutively on the coronal plane behind the optic chiasm with a slice thickness of 4 μm. Sections were placed on a slide treated with APES in advance, and baked in an oven at 60-62°C for 6-8 h. Then sections were stained with hematoxylin-eosin (HE) and AQP4, respectively.

HE staining (hematoxylin-eosin): Xylene dewaxing 10 min ×2 times; Dewaxed in ethanol 3 min ×2 times, 95% ethanol 2 min, 80% ethanol 2 min until natural water was hydrated; Gill's hematoxylin staining 10 min; Washed in natural water; Differentiated in 0.5% hydrochloric acid and ethanol for several seconds, rinsed with running waterand bluing using warm water; 95% ethanol 1 min, 0.5% ethanol eosin staining solution for 1-2 minutes; Differentiated in 80% ethanol, dehydrated in 95% ethanol 3 min ×2 times; Dehydrated in 100% ethanol 3 min ×2 times; Transparent by xylene 5 min ×2 times and mounted with neutral gum; Results were observed under a microscope

Immunohistochemical staining of paraffin sections of aquapori-4: Paraffin-embedded blocks were sliced, and dewaxed in xylene, 95%, 80% and 70% ethanol until the dehydration of natural water.Rinsed with distilled water; Antigen retrieval: high temperature and pressure repair was adopted (0.01 M sodium citrate buffer solution, PH: 6.0); Rinsed with distilled water and PBS, 5 min ×3 times; 3% H_2O_2 was used to block endogenous peroxidase for 10 min; Rinsed with PBS, 5 min ×3 times; An appropriate proportion of diluted primary antibodies were added, and sections were incubated overnight at 4°C (working concentration was 1:50). PBS replaced primary antibodies in the blank control group; Rinsed with PBS, 5 min ×3 times;
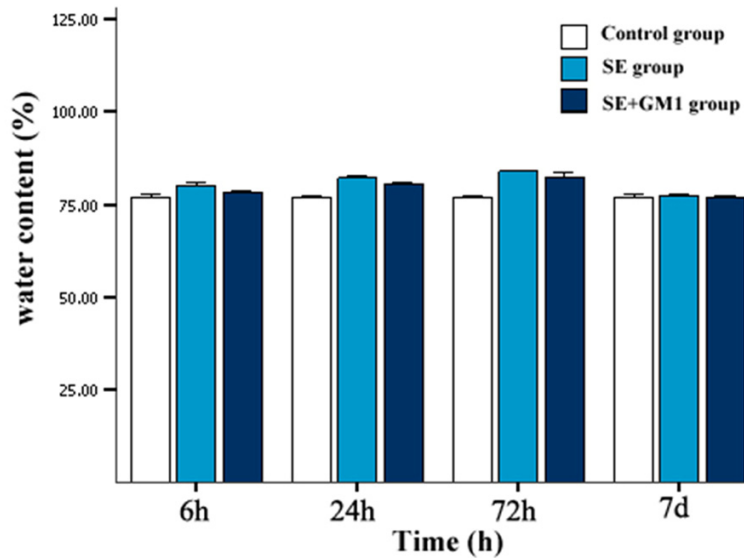
Figure 1. The brain water content changes after SE. Note: *P*<0.05 as the SE group compared with the control group at 6 h, 24 h and 72 h; *P*<0.05 as the SE group compared with the SE+GM1 group at 6 h, 24 h and 72 h; *P*>0.05 as groups compared with each other.

Incubated with goat anti-mouse (rabbit) IgG antibody-HRP polymer (Lot: 256810) at 37°C for 40 min; Rinsed with PBS, 5 min ×3 times; DAB color reagent was added to develop colors which underwent for 1-3 min, and the reaction was controlled under a microscope; The reaction was terminated by running water; Nuclei were counterstained with Harris' hematoxylin for 1 min, and dehydrated in 95% and 100% ethanol; Transparent by xylene and mounted with neutral gum; Result determination method: the cytoplasm of immunohistochemistry AQP4 positive cell was presented in yellowish brown.

Semi-quantitative detection of immunohistochemical APQ4 expressions: Pathological image analysis system was used for semi-quantitative detection of APQ4 expressions. That is color intensity of positive cells in the cerebral cortex on each slice was measured using pathological image analysis system. Five cortical visual fields (×200 times) were collected on each slice. Five average optical density values of the five fields were obtained from the automatic analysis of the system. The mean of these data represented the color intensity of the specimen, and then statistical analysis was conducted.

Statistical analysis: All data analysis was conducted using SPSS (version: 10.0). Measure-ment data were expressed as $\bar{x} \pm s$. Comparisons between two groups were examined using the LSD test, and the relationship between two variables was examined using the pearson correlation analysis. A *P* value less than 0.05 was considered statistically significant.

Results

The modeling results

There were 23 rats graded as I-III levels, 60 rats graded as IV-V, and 67 rats which died.

Behaviors of SE developing rats

The rats showed restlessness, scratching, face-washing behavior, single-limb clonus, wet dog shakes and balance disorders 10-30 min after an injection of PILO. Then the rats immediately showed tonic and clonic seizures in limbs accompanied with standing and forelimb clonus. The state of illness further reached the Level V, namely generalized tonic-clonic seizures with standing and falling (SE).

Morphological changes under a light microscope

HE staining in the control group showed that structures and layers of cortical neurons were clear without edema, degeneration and necrosis. Edema and denaturation occurred 6 h after SE. Edema and denaturation became significant, cytoplasm condensed and became dark, the volume of neurons reduced, and the membranes of neurons shrinked, the form of which was triangular or irregular 24-72 h after SE. Spaces appeared around neurons. On day 7, edema and necrosis of neurons were alleviated, and astrocytes proliferated. Changed in the SE+GM1 group was similar to that in the SE group, but to a lesser extent.

The distribution of AQP4 in the brain

AQP4 in the brain was widely distributed in astrocytes, ependymal cells, blood vessels and so on. There was no AQP4 expression in neurons.
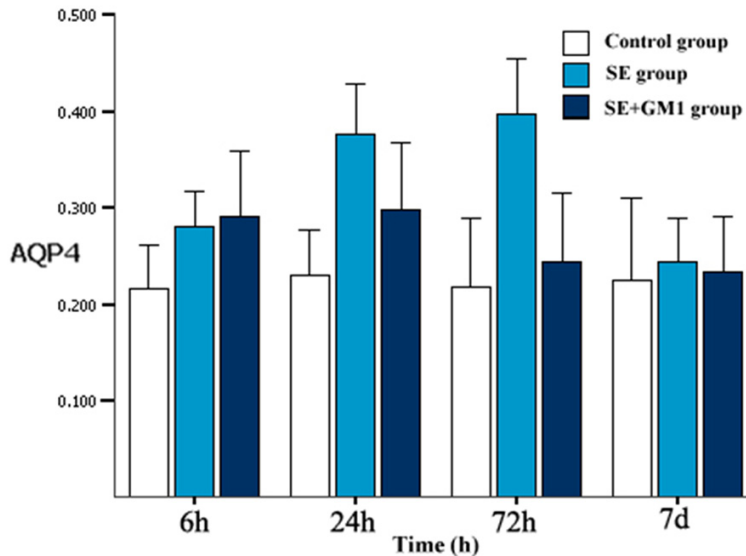
Figure 2. AQP4 expressions in the brain tissue after SE. Note: *P*<0.05 as the SE group compared with the control group at 6 h, 24 h and 72 h; *P*<0.05 as the SE group compared with the SE+GM1 group at 24 h and 72 h.

The brain water content changes

Compared with the control group, brain water contents in the developing rats in the SE group began to increase at 6 h (*P*<0.05), peaked at 72 h, and then gradually declined. On day 7, brain water contents in the SE group was higher than the control group without significant differences (*P*>0.05). The exression pattern of brain water contents in the SE+GM1 group was close to that in the SE group, and brain water contents in the SE+GM1 group were lower than the SE group at each time point (*P*<0.05) except on day 7 (**Figure 1**).

Immunohistochemical AQP4 expression results

AQP4 expressions in the SE group showed an upward trend over time: they began to rise at 6 h, significantly increased at 24 h, and peaked at 72 h. There were significant differences between the SE group and the control group (*P*<0.05). On day 7, the AQP4 expression in the SE group was still relatively high, but was not significantly different from the control group (*P*>0.05). AQP4 expressions were positively correlated with brain water content changes in the SE group (r=0.623, *P*<0.01). AQP4 expressions in the SE+GM1 group significantly decreased at 24 h and 72 h as compared with the SE group (*P*<0.05) (**Figure 2**).

Discussion

Pathophysiological mechanisms of SE are complex, and have not yet been fully elycidated. Under physiological conditions water in the brain tissue is tightly regulated. When convulsive seizures last for more than half an hour, especially one hour, decompensation of the cerebral circulation occurs. Subsequently a series of biochemical changes, such as ischemia, hypoxia, brain cell function failure, excessive release of excitatory amino acids, and acidosis, occur, leading to changes in the water balance and osmotic pressure. Water contents in cells and between tissues increase, causing BE [14, 15]. Changes in the aspects of pathology and imaging caused by the formation of BE after SE have been demonstrated [16-18]. BE following SE falls within the category of mixed BE. It begins with cytotoxic edema, and vasogenic edema occurs in the later period. In this experiment, water contents in the brain tissue in the SE group were significantly higher than the control group. This result indicated that BE forms after SE, which is consistent with the literature.

The formation mechanism of BE has not been yet entirely illustrated. Its pathogenesis is that factors such as blood-brain barrier damage, cerebral ischemia, intracranial venous hypertension lead to disorders of water transport and balance in the brain tissue as well as distribution imbalances of water and electrolytes inside and outside brain cell membranes. Currently, it is thought that there are 3 ways in the water transmembrane transport [19]: (1) channel proteins (aquaporin family, AQPs) on the cell membrane which were selectively permeable to water and can transport water molecules at a high speed; (2) cotransporters on the cell membrane which can perform coupled transmembrane transport of ions and water molecules; (3) diffusion through the lipid bilayer which is a slow way of transport. Among them, the first way is the most important form of transport. Abnormal water transmembrane

transport can result in water and electrolyte imbalance inside and outside the cells, causing BE and brain dysfunction. In addition, abnormal water transmembrane transport can bring about intracranial hypertension, aggravating the disease. When the condition is serious, brain herniation happens and even death occurs.

AQP mainly mediates the passive transport of free water across biological membranes and plays an important role in maintaining omeostasis of the intracellular and extracellular environments of cells. In all organs, the AQP4 content in the brain tissue is highest, and most widely distributed. Recently, literature in China and abroad has confirmed that AQP4 participates in the formation of BE under a variety of pathological conditions such as hypoxia-ischemia, inflammation, and trauma. In addition to the studies conducted by Manley et al., research conducted by Vajdaz et al. [20] on knockout mice (lacking a-syn) suffering from dystrophy showed that AQP4 expressions in knockout mice decreased in comparison with the control group. Meanwhile, due to the lack of a-syn, AQP4 in knockout mice was not properly positioned in the cell membrane, and thus could not play a biological activity. The occurrence of BE in knockout mice was delayed, and the state of the illness was relatively mild. The results indicated that the active decrease of AQP4 levels or activities can alleviate and even prevent the occurrence of BE. Ribeiro [21] has found that AQP4 expression was significantly up-regulated 1 h and 48 h after transient cerebral ischemia, and that changes in AQP4 expression was positively correlated with the degree of BE. Domestic scholars Lu Hong et al. [22] found that AQP4 expression began to increase in the ischemic penumbra (IP) 15 min after cerebral artery occlusion, and that the speed of increase was relatively high within 1 h, nearly presenting a linear increase. AQP4 expression showed a slow increase 1 h later. This trend continued until 24 h and was consistent with the occurrence of brain edema on MRI. At the same time, the relative apparent diffusion coefficient (rADC) in the IP showed a straight downward trend. The decline was most significant when the cell edema was most serious (<1 h), and showed a significant negative correlation with the AQP4 expression. The results confirmed that AQP4 upregulation is a key factor in the for-

mation of celledema. Other reports [23-25] have also proven that AQP4 expressions upregulated under pathological conditions such as brain tumors, inflammation, bleeding, trauma and cardiac arrest and that AQP4 upregulation is closely related to BE. Currently, there are relatively few reports in regard to AQP4 expression after SE. In this experiment, the immunohistochemical expression of AQP4 in the SE group showed an upward trend over time. The AQP4 expression began to increase at 6 h, and increased significantly from 24 h to 72 h. Furthermore, changes in AQP4 expressions were positively correlated with brain water contents. The above results indicated that AQP4 expression increases after SE, and that AQP4 is involved in the formation of BE. This finding is in agreement with reports which have found that AQP4 expression increases after ischemic and hemorrhagic BE. We inferred that AQP4 is involved in the formation of BE after SE, and that inhibition of AQP4 expression or activity can reduce BE.

GM1 is a neuroprotective agent which has multiple roldes. GM1 can stabilize the activities of a variety of enzymes on the cell membrane, correct ion imbalance outside and inside cells, reduce the production of intracellular Ca^+ load and free radicals, decrease the release and neurotoxic effects of excitatory amino acids, inhibit pathological excitement of NMDA receptors, and participate in the cellular signal transduction [25]. Exogenous GM1 has been proven to have definite protective effects on the brain in the treatment of cerebral ischemia, hypoxia and brain injuries. Meanwhile, studies have confirmed that exogenous GM1 can reduce BE in humans and rats after SE [26, 27]. Currently, studies on effects of GM1 on AQP4 expressions are relatively few. This experiment adopted GM1 to treat developing rats after SE, finding that brain water contents in the SE+GM1 group were significantly lower than the SE group, and also decreased in comparison with the SE group at the peaks of BE, that is 24 h and 72 h [28]. This indicated that GM1 can inhibit AQP4 expressions after SE, and reduce BE, thus exerting a protective effect on the brain. However, the specific mechanisms of how the AQP4 protein expression is affected are not clear. Further studies are needed to explore whether one or more signaling pathways are targeted.

Conclusion

As BE was aggravated in developing rats after SE, AQP4 expressions up-regulated, indicating that AQP4 is involved in the formation of BE following SE. Exogenous GM1 can inhibit AQP4 expressions after SE and alleviate BE following SE.

Acknowledgements

Disclosure of conflict of interest

None.

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