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Original Article

Effects of mannitol on hypoxic-ischemic brain edema and aquaporin-4 expression in neonatal rats

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Abstract: Brain edema after hypoxic-ischemic brain damage (HIBD) is a serious problem in neonates. Mannitol can mitigate edema; however, the molecular mechanism has not been fully elucidated. It has been reported that aquaporin-4 (AQP4), a member of the water channel protein family, plays a vital role in the development of brain edema. We therefore investigated whether mannitol might alleviate brain edema by regulating the expression of AQP4. In the present study, we used an established neonatal rat model of HIBD. A blood-brain barrier (BBB) permeability test revealed BBB leakage following HIBD, suggesting the vasogenic edema contributed to brain swelling during HIBD. Brain water content analysis indicated that mannitol could relief brain edema induced by HIBD after 6 h. In addition, qPCR showed that mannitol upregulated the expression of AQP4 in the first 48 h following HIBD induction. In conclusion, this study suggests that mannitol could alleviate brain edema after HIBD via upregulating the expression of AQP4. It may provide a novel insight into the treatment of HIBD.

Keywords: AQP4, brain edema, hypoxic-ischemic brain damage, mannitol, neonate

Introduction

Brain edema is a vital characteristic in the pathophysiological progress of hypoxic-ischemic brain damage (HIBD). Igor Klatzo classified brain edema into cytotoxic edema and vasogenic edema according to whether damage in the blood-brain barrier (BBB) happens [1]. Both types may result in acute intracranial hypertension and aggravate brain damage [2]. Therefore, it is essential to maintain normal brain water homeostasis after HIBD. The molecular mechanism of brain edema during HIBD as a hot area of research has not been fully elucidated.

Recent studies have shown that the aquaporin-4 (AQP4) is an important member of water channel proteins family and plays a key role in brain water homeostasis [3]. AQP4 is abundant in the central nervous system (CNS), especially in astrocyte foot processes and capillary endothelial cells on the BBB [4], a diffusion membrane which exerts a vital role in inhibiting unwanted substances entering into the brain. However, the roles of AQP4 in brain water homeostasis and brain edema are complicated and often depend on the form of edema. Several researches have proved that in the model of cytotoxic edema, AQP4 deletion could reduce brain swelling by limiting water entry into cells [5]. Accordingly, overexpression of AQP4 was found to accelerate cytotoxic brain edema [6]. While in vasogenic edema, AQP4 deletion promoted the formation of brain edema by reducing brain water clearance [7]. Although there are numerous literatures have reported the important roles of AQP4 in brain edema, little is known about the relationship between brain edema after HIBD and the dynamic changes of AQP4.

Mannitol is a hyperosmotic solution and can efficiently reduce intracranial pressure, therefore, it is used clinically to alleviate brain edema [8]. However, there is little research on the rela-

Table 1. The primers for AQP4 and β-actin

Genes	Primers	Sequences (5' to 3')
AQP4	Forward primer	GCTGTGATTCCAAACGGACT
	Backward primer	CAGTGGTTTTCCCAGTTTCC
β-actin	Forward primer	CCCATCTATGAGGGTTACGC
	Backward primer	TTTAATGTCACGCACGATTTC

tionship among mannitol, brain edema and AQP4. In this study, the HIBD animal model was successfully built to investigate the effect of mannitol on brain edema and AQP4 expression. Brain water contents at different time points were examined to assess the level of brain edema. qRT-PCR was performed to analyze the expression of AQP4. The results revealed that mannitol alleviated brain edema caused by HIBD via regulating the expression of AQP4. This study investigated the effect of mannitol on brain edema and AQP4 expression in HIBD animal model. Furthermore, it will provide a novel insight into the treatment of HIBD in neonates.

Materials and methods

Animals

A total of 216 neonatal Sprague-Dawley (SD) rats (10-20 g) at the age of 7 days were obtained from the Animal Experiments Center of Xi'an Jiaotong University. The rats were housed in a specific pathogen free environment with free access to water and chow. The SD rats were randomly divided into 3 groups: sham operation group (n=72), the right common carotid artery (CCA) was separated without ligated; HIBD group (n=72), the HIBD animal model was constructed; mannitol group (n=72), after HIBD animal model was completed, the rats received an intraperitoneal injection of 20% mannitol at a dose of 1 g/kg. Each group was divided into 6 subgroups according to different time points of their termination (0 h, 6 h, 12 h, 24 h, 48 h, 72 h).

The experimental procedures and animals used in this study were approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. All efforts were made to minimize suffering.

Construction of HIBD animal model

HIBD animal model was constructed as our previous study [9]. Briefly, the rats were anesthetized with chloral hydrate, right CCA was carefully separated and ligated with 7-0 surgical silk. After which the animals were kept in normoxic environment for 1 h, then exposed to 1 hour period of hypoxia (mixture of 92% nitrogen and 8% oxygen). Then the rats were returned to their dams until sacrificed.

Detection of BBB permeability

BBB permeability was detected by using Evans Blue (EB, 2%) as a tracer. Rats were injected with 2 ml/kg EB and physiological saline solution to heart cavity 2 hours before decapitated. Brain tissues were obtained immediately and homogenized with 50% trichloroacetic acid. After centrifuging at 15,000 rpm for 20 min, the EB content was detected by a spectrophotometer at 600 nm. The calculation of EB was based on the standard curve and the data were expressed in μg/g wet brain tissue.

Determination of brain water content

The rats were sacrificed by decapitation, fresh brain tissues were removed and weighed immediately to measure wet weight. Then dried these tissues in an oven at 100°C for 24 h and reweighed to measure dry weight. Water content of brain tissue was calculated as the formula: [(wet weight-dry weight)]/wet weight ×100%.

qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA) and reverse transcribed into complementary DNA (cDNA) using PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). Realtime quantitative PCR analysis was performed applying SYBR Premix Ex Taq™ II (Takara, Dalian, China) according to manufacturer's instruction. The expression level of AQP4 was analyzed with melting curve analysis and normalized to internal β-actin. The primers for AQP4 and β-actin were provided by Takara (**Table 1**).

Statistical analysis

Data were expressed as mean ± standard deviation (SD), statistical analysis was performed applying SPSS 17.0 (SPSS Inc). The differences between two groups were analyzed using independent 2-sample *t* test. The differences among multiple groups were analyzed

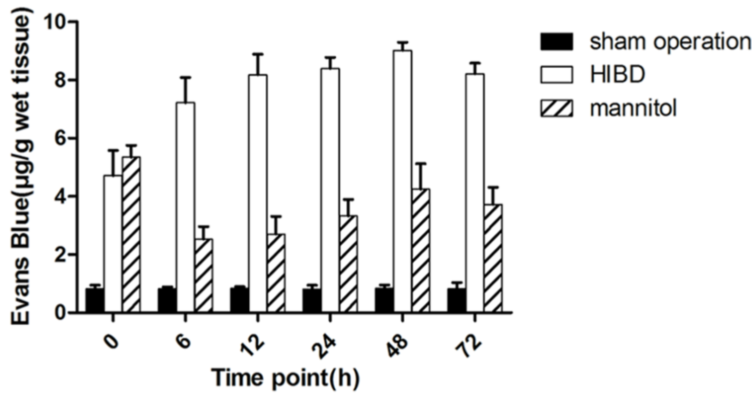

Figure 1. EB content in the three groups. The EB content in sham operation group, HIBD group and mannitol group at different time points after treatments was detected. The sham operation group showed no significant difference at each time point (P>0.05). The HIBD group showed significantly higher EB level than the sham operation group at each time point (P<0.05). The mannitol markedly decreased EB content except for 0 h (P<0.05).

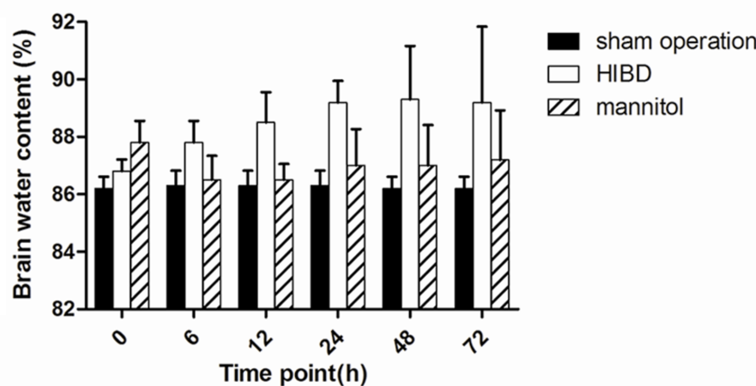

Figure 2. Brain water content in the three groups. The level of brain water content in the sham operation group had no significant difference at each time point (P>0.05). HIBD group showed significantly higher water content than the sham operation group at each time point (P<0.05). The mannitol decreased the water content almost to baseline.

using one-way ANOVA analysis. *P* value <0.05 was considered statistical significance.

Results

Detection of BBB permeability

To determine the effects of HIBD and mannitol on BBB, we detected BBB permeability by EB staining. As shown in **Figure 1**, The EB content in sham operation group showed no significant difference at each time point (P>0.05). However, in HIBD group, the EB content increased from 0 to 48 h, and then decreased. The level was significantly higher than the sham operation group at each time point (P<0.05). While in the mannitol group, the EB content decreased from 0 h to 6 h and then increased slightly. The level at each time point was significantly lower than that of HIBD group except for 0 h (**Figure 1**).

Brain water content

Brain wet-dry weight ratios were calculated to determine water content in different groups. The level of brain water content in the sham operation group had no significant difference at each time point (P>0.05). Water content in HIBD group rose sharply from 0 h to 48 h, and decreased at 72 h. The level was significantly higher than the sham operation group at each time point (P<0.05). Conversely, in the mannitol group, the brain water content at each time point was significantly lower than that of HIBD group except for 0 h, almost reached to baseline. In the comparison between the sham operation and mannitol group, no significant difference was found (P>0.05) except for 0 h (**Figure 2**).

AQP4 mRNA expression

Quantitative real-time PCR was performed to identify the expression level of AQP4 mRNA (**Figure 3**). No distinct difference at each time point in the sham operation group was observed. In the HIBD group, the extremely low expression level was at 48 h, while the peak value was at 72 h. At 0 h, 6 h and 72 h, AQP4 mRNA expression in HIBD group was significantly higher than in sham operation group (P<0.05), but significantly lower at the other three time points. In mannitol group, the maximum expression of AQP4 mRNA was presented at 0 h, and the minimum value was presented at 72 h. When mannitol group was compared with the sham operation and HIBD

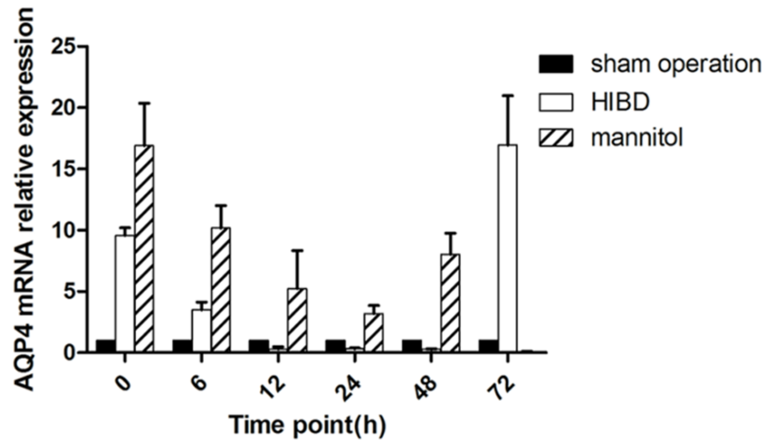

Figure 3. AQP4 expression in the three groups. No distinct difference at each time point in the sham operation group was observed (P<0.05). The HIBD group showed significantly higher AQP4 level than sham operation group at 0 h, 6 h and 72 h (P<0.05), but significantly lower at the other three time points. In mannitol group, the AQP4 expression was significantly higher than sham operation and HIBD group except at 72 h (P<0.05).

groups, the expression was significantly higher at each time point except at 72 h (P<0.05).

Discussion

In the present study, we used a HIBD animal model to investigate the effect of mannitol on brain edema after HIBD and its mechanisms via AQP4. Since the roles of AQP4 are totally different in cytotoxic edema and vasogenic edema, it is important to find out the major type of brain edema during HIBD. It has been reported that cytotoxic edema without obvious BBB damage is the major form in the early period of hypoxia or ischemia. With the BBB damaging, vasogenic edema occurred in the later period of hypoxia or ischemia [10]. Our research showed that hypoxia-ischemia (HI) increased brain water content and BBB permeability from 0 h, which proved that in the beginning stage of HI, the vasogenic edema had begun to form and continued to the later period. The possible reason may be the simultaneous occurrence of hypoxia and ischemia in our model aggravated the damage of BBB thus accelerated the progress of vasogenic edema.

In HIBD group, we found that the changing trends of the brain water content and BBB permeability at different time points from 0 h to 72 h were similar, the peak level were at 48 h. It is indicated that hypoxia and ischemia induce brain edema by damaging the BBB. However, the brain water content and BBB permeability decreased slightly at 72 h, which suggested that the effect of HIBD on brain appears to abate after 48 h. In order to illustrate the molecular mechanism, we analyzed the expression of AQP4 at different time points after HI. We observed that in the brain edema acceleration period (from 0 h to 48 h), the expression of AQP4 gradually decreased and achieved a lowest level at 48 h. Although the reason for the decrease of AQP4 after HI is unclear, it had been confirmed that AQP4 was in charge of water elimination in vasogenic edema. Therefore, we speculate that the decrease of AQP4 after HI limited the water clearance thus accelerated the progress of brain edema. The following upregulation of AQP4 may be explained as a self-protective reaction to eliminate excess water in brain and prevent the development of brain edema.

While in the mannitol group, the results showed that mannitol had a significant effect on decreasing BBB permeability and alleviating edema induced by HI, especially at 6 h after operation, the brain water content almost reached to baseline. However, in the early stage of HI, mannitol seemed to increase the BBB permeability and thus aggravated edema. It is indicated that mannitol was capable of opening BBB transitorily and accelerated the early period of brain edema. From the analysis of AQP4, we found that mannitol markedly upregulated the expression of AQP4 but downregulated the brain water content, suggesting that the alleviation of brain edema was at least in part associated with the high expression of AQP4. It can also be explained as the AQP4 contributed to water clearance in vasogenic edema. However, the mechanism for the regulating effect of mannitol on AQP4 is not fully understood. Recent studies have demonstrated that hyperosmotic stress could increase the expression level of AQPs. For instance, Hajime Arima *et al* found that mannitol was

able to increase the expression of AQP4 and AQP9 in cultured rat astrocytes and brain cortex. Besides, Hajime Arima also confirmed that the neuroprotection of mannitol was associated with the activation of p38 MAPK pathway in astrocytes [11]. However, little research has been done on the relationship between mannitol and AQP4 in neonatal brain.

In conclusion, the present study proves that mannitol relieves brain edema after HIBD by upregulating the expression of AQP4. More functional research of AQP4 is needed to further illustrate the mechanism after HIBD.

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Disclosure of conflict of interest

None.

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