Effect of hyperbaric oxygen preconditioning on peri-hemorrhagic focal edema and aquaporin-4 expression

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Abstract. The aim of the present study was to investigate the effect of hyperbaric oxygen preconditioning (HBO-PC) on peri-hemorrhagic focal edema and aquaporin-4 (AQP-4) expression in an experimental intracerebral hemorrhage (ICH) rat model. Sixty-six Sprague Dawley® rats were divided into three groups: The sham-surgery group (SHG; n=6); the control group (A-ICH; n=30), in which the rats were injected with autologous blood; and the experimental HBO-PC group (P-HBO; n=30). The rats underwent brain edema and AQP-4 detection at 5 postoperative time-points (24, 48 and 72 h and 5 and 7 days). The water content in the brain tissues of the A-ICH animals was higher than that in the brain tissues of the SHG rats at each time-point (P<0.05), and the edema in the P-HBO was significantly more severe 24 and 48 h post-operatively than that at 7 days postoperatively (P<0.05). The difference between the P-HBO and A-ICH was significant at 48 and 72 h postoperatively (P<0.05). AQP-4 was expressed in the post-hemorrhagic rat brains of all groups; the SHG animals exhibited low expression, while the A-ICH animals exhibited an increased expression 24 h postoperatively. In the A-ICH, expression peaked at 48 h postoperatively and began to decrease gradually after 72 h. At the 7-day time-point, the expression level in the A-ICH was closer to but still higher than that of the SHG animals (P<0.05). The differences between the P-HBO and A-ICH animals at the postoperative 24-h, 48-h and 7-day time-points were statistically significant (P<0.05). In conclusion, HBO-PC may downregulate AQP-4 expression to reduce the intracerebral edema, thus strengthening tolerance to ICH and protecting the nerves.

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Introduction

Intracerebral hemorrhage (ICH) refers to a non-traumatic cerebral parenchymal hemorrhage with high mortality and morbidity rates, which can have a considerable impact on human health. Post-ICH secondary cerebral edema damages the blood-brain barrier, triggers disorders of the sodium pumps in the brain, causes cellular edema, increases intracranial pressure and eventually leads to nerve cell necrosis (1,2). As a result, post-ICH edema is the main cause of disease progression and mortality. Thus, effective control of cerebral edema could significantly reduce ICH-induced neurological damage.

Hyperbaric oxygen (HBO) therapy refers to the exposure of the body to a high-pressure environment (>1 standard atmospheric pressure), so that the patient breathes in HBO or hyperbaric mixed oxygen (97% O_2 + >3% CO_2), in order to achieve therapeutic effects against various diseases (3). Numerous animal experiments have confirmed that HBO preconditioning (HBO-PC) can significantly reduce hypoxic-ischemic injuries in the brain, spinal cord and myocardium (4,5); however, few reports have described its application in ICH treatment (6,7).

Aquaporin-4 (AQP-4) is a membrane protein that mediates the transmembrane water transportation of various types of cells. The protein is composed of four active subunits that form a heterotetrameric structure, as confirmed by three-dimensional technology (8). AQP-4 is mainly distributed in astrocytes, and the cells that most intensively express the protein lie on the glial limiting membrane, which is formed by the subarachnoid astrocyte foot processes and the surface of the perivascular astrocytes. AQP-4 can also be expressed in the ependymal cells, choroid plexus and pia mater, as well as in the paraventricular and supraoptic nucleus of the hypothalamus (9). AQP-4 has been found to be the only cell membrane transportation protein that is permeable to water molecules and other small molecules. It comprises, therefore, the structural basis of water transportation and regulation among the cerebrospinal fluid, glial cells and blood vessels, has a close association with the development of the blood-brain barrier and plays a key role in the regulation of the cerebral water balance (10). It remains unclear whether

HBO reduces cerebral edema by affecting the AQP-4 expression in the brain tissues of patients with ICH.

The present study examined the peri-hematoma edema and AQP-4 expression in experimental ICH rats following HBO-PC and aimed to investigate the effects and mechanism of HBO-PC in the treatment of ICH.

Materials and methods

Animals. Healthy adult male Sprague Dawley® rats (n=156), weighing 350-380 g, were provided by the Experimental Animal Center of Hebei Medical University (Shijiazhuang, China). Prior to the experiment, the rats were cage-bred separately in the Experimental Animal Center of the Second Hospital of Hebei Medical University (Shijiazhuang, China) at a constant temperature of 20-25˚C and with a standard diet and drinking water available *ad libitum*. The present study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

Animal grouping. Sixty-six of the rats were randomly divided into three groups: The sham-surgery group (SHG; n=6); the control group (A-ICH; n=30), in which the rats were injected with autologous blood; and the experimental group (P-HBO; n=30), in which the rats underwent a 5-day period of HBO-PC before being prepared as an ICH model. The latter two groups were then randomly divided into five subgroups, namely the postoperative 24-h, 48-h, 72-h, 5-day and 7-day subgroups, with 6 rats in each subgroup.

ICH model. The preparation of an autologous blood-injected ICH model was performed as previously described (11). Following anesthesia with chloral hydrate, the rats were fixed on a stereotactic frame (Stoelting Co., Wood Dale, IL, USA); the bregma was exposed and a small hole was then drilled 0.5 mm anterior to the bregma and 3 mm to the right of the midline. A total of 50 μl arterial blood was subsequently extracted with a micro-syringe and inserted into the drilled hole. The needle depth was 5.8 mm (to approximate the position of the caudate nucleus), and the injection lasted 10 min and was followed by needle-standing for 5 min. Bone wax was used to seal the pinhole and the skin was disinfected and sutured. The same method was used for the SHG model preparation but an equal volume of saline, instead of blood, was used.

HBO-PC. Prior to the preparation of the ICH model, a single infant oxygen chamber (type YL0.5/1.2; Wuhan Second Ship Design Institute, Wuhan, China) was used to perform HBO exposure on the rats in the experimental group. The pressurization time was 15 min, the pressure was regulated at 0.10 MPa and the oxygen concentration was maintained at >90% for 60 min of oxygen aspiration. The decompression time was 15 min and the cabin temperature was maintained at ~24˚C. HBO-PC was performed once a day for 5 consecutive days and then the ICH model was prepared using the aforementioned method.

Assessment of neurological function. Twenty-four hours after the successful preparation of the animal models, the behavior of the rats of each group was scored according to the improved Longa classification method (12). The scores were as follows: 0 points, no symptoms of neurological deficit; 1 point, inability to extend the contralateral forelimb; 2 points, tonic flexion of the contralateral forelimb; 3 points, mild circling around the contralateral side; 4 points, severe circling around the contralateral side; 5 points, falling toward the contralateral side. Scores >1 point indicated the successful preparation of the model.

Determination of cerebral water content. The rats in each group were sacrificed at the appropriate time-points by spinal dislocation and the wet and dry weight method was used to measure the water content in the peri-hemorrhagic cerebral tissues. Following the removal of the frontal pole, a 2-mm-thick sample of brain tissue was extracted from the lesion side for the purpose of determining the water content. The brain tissue was placed into pre-weighed tin foil (A), and the combined weight of the foil and brain tissue (B) was obtained. The result of B-A was the wet weight of the brain tissue. The brain tissue was then wrapped with the tin foil and placed into an electric oven (WH-43; Tianjin Taisite Instrument Co., Ltd., Tianjin, China) and dried at 100˚C for 24 h. The brain tissue and foil (C) were then reweighed upon returning to room temperature. The result of C-A was the dry weight. Finally, the data were entered into the following equation: Brain water content = (wet weight-dry weight)/wet weight x 100% [(B-C)/(B-A) x 100%].

Determination of AQP-4. Following anesthesia with chloral hydrate, the rat brain tissues were obtained, fixed in 4% paraformaldehyde, rinsed in 0.01 M phosphate-buffered saline, dehydrated with a conventional ethanol gradient, hyalinized with xylene and embedded in paraffin. A tissue slicer (Leica Microsystems, Wetzlar, Germany) was then used to prepare 5-μm tissue sections. After enzyme closure with 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) and antigen retrieval using citrate buffer (Sigma-Aldrich), the staining was performed using the immunohistochemical Avidin Biotin Complex method using a primary rabbit anti-rat polyclonal AQP-4 antibody (cat. no. sc20812; dilution, 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (cat. no. ab67203; dilution, 1:200; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's instructions. PBS was used instead of primary antibody as a control. Three brain slices were selected for each rat and 5 different randomly selected fields of view were observed with a medical optical microscope (Olympus Corp., Tokyo, Japan) at x400 magnification. The positive cells (positive appearance of AQP-4 exhibited as brownish-yellow cytoplasm) were counted in order to calculate the rate of positive cells in the brain tissues.

Statistical analysis. All data were entered into the computer to generate a database, and SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant

Table I. Postoperative cerebral water content among the different groups.

Group	24 h (%)	48 h (%)	72 h (%)	5 days (%)	7 days (%)
SHG	76.83±2.61	76.83±2.61	76.83±2.61	76.83±2.61	76.83±2.61
A-ICH	83.14±3.29[a]	86.12±2.10[a]	85.58±4.74[a]	82.78±3.84[a]	81.91±2.67[a]
P-HBO	81.31±3.03[a]	83.04±3.43[a,b]	80.86±4.04[a,b]	79.07±2.03[a]	77.64±4.07

Data are presented as the mean ± standard deviation. [a]Compared with SHG, P<0.05; [b]compared with A-ICH, P<0.05. SHG, sham-surgery group; A-ICH, intracerebral hemorrhage group; P-HBO, hyperbaric oxygen preconditioning group.

Table II. Aquaporin-4 expression among the different groups.

Group	24 h (%)	48 h (%)	72 h (%)	5 days (%)	7 days (%)
SHG	39.45±5.67	39.45±5.67	39.45±5.67	39.45±5.67	39.45±5.67
A-ICH	45.06±3.36[a]	52.76±5.16[a]	49.07±2.80[a]	47.59±4.82[a]	44.68±3.77[a]
P-HBO	44.27±4.30[a,b]	48.72±3.96[a,b]	45.30±4.01[a]	43.29±4.03[a]	40.05±2.11[b]

Data are presented as the mean ± standard deviation. [a]Compared with SHG, P<0.05; [b]compared with A-ICH, P<0.05. SHG, sham-surgery group; A-ICH, intracerebral hemorrhage group; P-HBO, hyperbaric oxygen preconditioning group.

difference. Analysis of variance was used for the analysis of the mean values of the measurement data of multiple groups.

Results

Success rate of animal model establishment. The establishment of the ICH model had a 60% success rate, and the experimental animals typically died 48-72 h after the lesioning. The rats that died were replenished in a timely manner to ensure that the number of rats in each experimental group did not change.

Scoring of neurological function. The mean 24-h postoperative neurological scores of the SHG, A-ICH and P-HBO were 0, 4.12±0.41 and 3.91±0.37, respectively. The A-ICH animals exhibited significant neurological dysfunctions compared with the SHG animals (P<0.05); however, no significant differences where observed in the degree of neurological dysfunction between the HBO-PC and A-ICH (P>0.05).

Cerebral water content. The water content in the brains of the A-ICH animals was higher than that in the brains of the SHG animals at all time-points (P<0.05). In the A-ICH, the cerebral edema was most obvious 48 h postoperatively. Despite the fact that the edema showed a tendency towards alleviation as the time passed, the water content remained significantly higher in the A-ICH than that in the SHG 7 days postoperatively (P<0.05). The cerebral edema in the P-HBO was significantly more severe 24 and 48 h postoperatively than that at 7 days postoperatively (P<0.05). After 48 h, the edema gradually reduced and essentially returned to the level of SHG on postoperative day 7. Compared with the A-ICH, the edema was reduced in P-HBO animals, particularly at 48 and 72 h postoperatively, when the difference between the groups was significant (P<0.05) (Table I).

AQP-4 expression. Following the ICH, AQP-4 was expressed in the brain tissues of all groups. The lowest AQP-4 expression was observed in the SHG. The AQP-4 expression of the A-ICH started to increase 24 h postoperatively, peaked 48 h postoperatively and began its gradual decrease at 72 h. The expression in the A-ICH was close to but still higher than that of the SHG on postoperative day 7 (P<0.05). The AQP-4 expression in the P-HBO at each time-point was consistently lower than that in the A-ICH, with significant differences between the two groups at 24 h, 48 h and 7 days postoperatively (P<0.05) (Table II and Fig. 1A and B).

Discussion

The results of the present study showed that the success rate of ICH establishment was 60%, which was lower than the 71 and 79% reported previously (11,12). It is generally believed that the factors affecting the success rate of modeling are the following (13): The amount of narcotic drugs and blood injected, the insertion depth of the micro-syringe needle, the time and speed of liquid injection and the living conditions of the animals. The mortality rate in the present study was high and the animals typically died 48-72 h after the model preparation, at which time the cerebral edema was at its most severe form. Since this experiment was carried out in the hot summer months and there was a 30-min distance from the animal laboratory to the HBO treatment site, it may have been that, besides the aforementioned laboratory factors, the environmental factors were the key reason for the high levels of animal mortality in the present study.

With regard to the treatment of ICH, common clinical strategies comprise medical and surgical approaches; however, HBO therapy has recently been started to be assessed in trials as a potential ICH treatment method. In a previous clinical study HBO has exhibited significant effects in relieving post-ICH

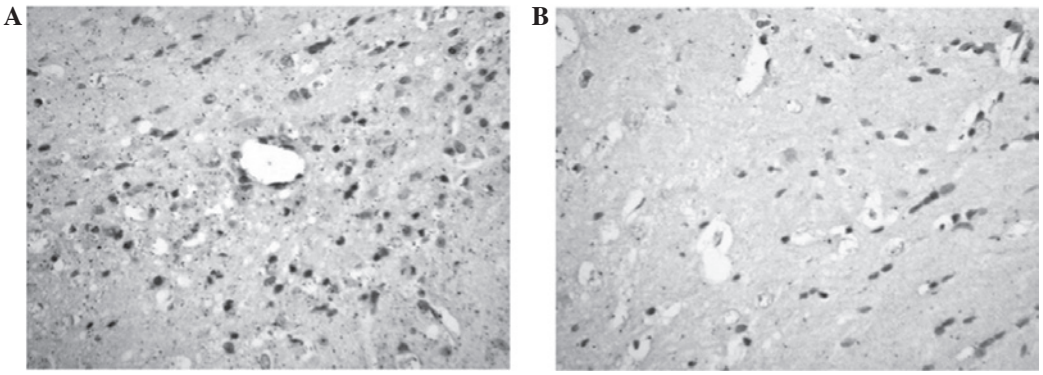

Figure 1. Aquaporin-4 expression 24 h postoperatively in the (A) control and (B) hyperbaric oxygen preconditioning groups (magnification, x400).

secondary cerebral edema; however its specific mechanism of action remains unclear (14). Cerebral edema is a common pathological alteration that follows cerebral cell injuries induced by trauma, hemorrhage, ischemia and cancer, and is a key factor affecting the prognosis and lives of the patients. The cerebral edema formation-related factors have been suggested to include the following: Toxic effects of hemoglobin on the brain tissues generated by the brain damage-induced red blood cell lysis and rupture; neuronal apoptosis and proinflammatory reactions induced by prothrombin activation; complement activation effects caused by the inflammatory response; and water balance disorders in the brain tissues caused by damage to the blood-brain barrier (15-17). In short, the formation of cerebral edema is a complex process, which includes and is influenced by a variety of factors. In 2000, Manley *et al* (18) used AQP-4 gene-knockout mice for the water intoxication experiment, and the survival rate of gene-knockout mice was found to be significantly higher than that of the mice of the control group. The brain water content and the angioedema in the capillary ultrastructural observation of these animals were lower than those in the control group, which confirmed the association between AQP-4 and cerebral edema.

AQP-4 is a functional protein, which was first separated from the red blood cell Rh proteins by Agre in 1998 (19) and was confirmed to be a membrane protein that could mediate extra- and intracellular water transportation in 1992 (20). The characteristic distribution of AQP-4 in brain tissues is that it is most intensively expressed on the glial limiting membrane, formed by the astrocyte foot processes and the surface of the perivascular astrocytes (21). AQP-4 exhibits a highly polar expression distribution on the glial foot process membranes, and its density in the pia mater region has been shown to be several times the density in the neuropil (22,23). The distribution of AQP-4 on the brain tissue membrane has been found to be consistent with the polarity distribution of K^+ channels. The expression sites of AQP-4 in the choroid plexus epithelial cells, periventricular ependymal cells and pia mater are consistent with its reabsorption sites (24). The above distribution characteristics of AQP in the brain could suggest that AQP-4 is the structural base of water transportation between the cerebrospinal fluid and cells and that it plays a role in maintaining the intracellular and extracellular balance of water and K^+ concentrations and participating in the regulation of osmotic pressure. Thus, AQP-4 is the key factor that affects the water and electrolyte balance in the central nervous system; this finding provides a theoretical basis for further studies on the association between AQP-4 brain edema.

HBO therapy, an effective means of treating cerebral edema, was previously reported to reduce secondary cerebral edema caused by subarachnoid hemorrhages and traumatic brain injury through the inhibition of AQP-4 expression; however, it remained unclear whether HBO therapy could affect the AQP-4 expression (25) in post-ICH cerebral tissues. As a special type of HBO therapy, HBO-PC is usually used in the study of the incidence of high altitude reactions. It has been shown that HBO-PC does not only improve the body's capacity for oxidation resistance and reduce the incidence of high-altitude reactions, but it also plays a neuroprotective role in rats with altitude-induced traumatic brain injuries and improves their neurological functions (26). Other studies have shown that HBO-PC can enhance the ischemic tolerance of the spinal cord and promote functional nerve recovery following spinal cord injury (27-29). HBO-PC has been increasingly used in clinical surgeries as a novel method that could improve the success rate of surgery. For example, the application of conventional HBO-PC on surgical patients several days before their surgery could effectively reduce the side effects of anesthesia and improve the hypoxic tolerance of the heart, brain and other vital organs. It has also been shown that HBO-PC can improve myocardial function following coronary bypass surgery and reduce myocardial injury (30). A clinical study confirmed that HBO could significantly improve the early clinical symptoms of allergic vasculitis, and HBO-PC could effectively prevent or reduce its complications (31).

Regarding the mechanism underlying the protective effect of HBO-PC in hypoxic-ischemic encephalopathy, the results of previous experimental studies (32-36) on an ICH animal model under a high-pressure oxygen environment revealed that HBO-PC could reduce apoptosis in the early stage of ICH and inhibit the apoptotic transformation of damaged brain cells in the late stage of ischemia. Its brain-protective effect was associated with the upregulation of the brain-derived neurotrophic factor expression level, as well as with the inhibition of mitogen-activated protein kinase p38 activities. Another study suggested that HBO-PC could reduce post-cerebral hypoxic nerve damage by upregulating the activities of antioxidant enzymes, such as catalase, superoxide dismutase and cellular hypoxia-inducible factor-1α, among others (34). This could promote the generation of erythropoietin in the cerebral cortex and hippocampus, change the permeability of the blood-brain

barrier, reduce cerebral edema and, thus, promote the recovery of neurological function. In the aforementioned studies, the role of HBO-PC in the treatment of hypoxic-ischemic encephalopathy was further clarified; however, reports on the application of HBO-PC in ICH are still rare. Qin *et al* (37,38) applied HBO-PC to experimental ICH rats and found that the activation of p44/42 mitogen-activated protein kinase in the brain tissue was associated with the degree of cerebral edema. HBO-PC was shown to be involved in the synthesis of heat shock proteins by activating p70 S6 kinases, thereby inducing the protective effect in post-ICH brain tissues (38). This study introduced novel ideas for the effect of HBO in ICH.

The present experimental results showed that, despite the reduction in the postoperative neurological dysfunction of the P-HBO rats at 24 h after ICH, the difference between the neurological dysfunction of the P-HBO and A-ICH rats was not significant, indicating that HBO-PC could not alleviate the symptoms at the onset of ICH; however, the cerebral edema and AQP-4 expression levels around the hemorrhagic focus in the P-HBO were significantly lower than those in the A-ICH at various time-points, suggesting that HBO-PC downregulated AQP-4 expression. This downregulation reduced the cerebral edema, thus playing a neuroprotective role and strengthening the resistance to ICH. The present study provided evidence for the clinical application of HBO-PC in the prevention of ICH-associated diseases; however, large, randomized, controlled studies are required for the confirmation of the treatment effects and mechanisms of HBO-PC against ICH.

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