Expression of Aquaporin-1 and Aquaporin-5 in a Rat Model of High-Altitude Pulmonary Edema and the Effect of Hyperbaric Oxygen Exposure

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Abstract

Objective: To investigate the therapeutic roles of hyperbaric oxygen exposure on high-altitude pulmonary edema and to determine whether aquaporin-1 and aquaporin-5 were involved in the pathogenesis of HAPE in rats.

Methods: Rats were divided into 5 groups: The control group, the HAPE group (HAPE model), the HBO group (hyperbaric oxygen exposure), the NBO group (normobaric oxygen exposure), and the NA group (normal air exposure). Western blot and real-time PCR were used to analyze the pulmonary expressions of AQP1 and AQP5. The wet-to-dry (W/D) weight ratio and the morphology of the lung were also examined.

Results: The lung W/D weight ratio in the HAPE group was increased compared with the control group. The injury score in the HBO group was noticeably lower than that in the control group. The mRNA and proteins expressions of AQP1 and AQP5 were significantly downregulated in the HAPE group.

Conclusions: Oxygen exposure alleviated high-altitude hypobaric hypoxia-induced lung injury in rats. Additionally, HBO therapy had significant advantage on interstitial HAPE.

Keywords
high-altitude pulmonary edema, hyperbaric oxygen therapy, aquaporin-1, aquaporin-5, lung wet-to-dry weight ratio, lung morphology

Introduction

High-altitude pulmonary edema (HAPE) is a life-threatening disorder among climbers and tourists who ascend to altitudes >2500 m. It was first described in South American high altitude dwellers who returned from a sojourn at low altitude and subsequently in acclimatized lowlanders.2

HAPE usually develops within the first 2-5 days after the arrival at high altitude and presents with cough, dyspnea, tachycardia, and even with orthopnoea and pink sputum at advanced stage.3-5 The incidence of HAPE among trekkers in the Himalayas and climbers in the Alps ascending at a rate of >600 m/day is around 4%.6,7 In the alpine setting, when an altitude of 4559 m was reached within 22 h, the incidence increased to 7% in mountaineers without a history of radio-graphically documented HAPE and to 62% in mountaineers with such a history.8

The pathogenesis of HAPE has not been fully elucidated. Recent evidence suggests that aquaporins (AQP) may play an important part in fluid clearance and edema formation in the lung after acute lung injury (ALI).9 AQP are a family of water-selective channels that increase plasma membrane water permeability and provide a route for rapid fluid movement.10 AQP are expressed in many tissues, such as the kidney, eye, and
Some clinical studies reported HBOT in HAPE, but few investigations may benefit from HBOT with hyperbaric hyperoxia. Altitude of 6000 m for 24 h in a hypobaric chamber to produce a hypobaric air (NA) group. All rats were exposed to a simulated normobaric oxygen therapy (NBOT) on lung edema using wet/dry (W/D) weight ratios and histological studies of lung tissue. We also observed changes in mRNA and protein expressions of AQP1 and AQP5 in the lung tissues, and further investigated the role of AQP1 and AQP5 in HAPE.

### Materials and Methods

#### Ethical Approval of Study Protocol

This study was approved by the Ethical Committee of the Animal Experimentation of Capital Medical University (Beijing, China) and performed in accordance with Guidelines for Ethical Review of Experimental Animal Welfare (GB/T 35892).

#### Study Design

Rats were divided into the 5 groups: control group, HAPE group, HBO group, normobaric oxygen (NBO) group, and normobaric air (NA) group. All rats were exposed to a simulated altitude of 6000 m for 24 h in a hypobaric chamber to produce a rat model of HAPE except for the control group. Immediately after being taken out to the ambient atmosphere, HAPE rats were anesthetized by administration of 10% chloral hydrate (5 mL/kg, i.p.). HBO rats were treated with HBO for 1 session; NBO rats were treated with normobaric oxygen for 2 h; and rats in the NA group were exposed to the ambient atmosphere without any treatment for 2 h.

At the different time-points mentioned above, rats were euthanized and thoroughly exsanguinated before their lungs were excised en bloc. The left lung lobe was used for determination of the W/D ratio (which was used as an index of water content in the lung). The right lower lobe was harvested for histopathology evaluation, and the right upper lobe was collected for molecular analyses.

#### Animals

Adult male Wistar rats (220-250 g) were obtained from the Military Medical Science Academy of the People’s Liberation Army (Beijing, China). Rats were maintained at 25 ± 1°C under a 12-h:12-h light-dark cycle. They had ad libitum access to food and water. Forty-weight rats were randomly assigned into 5 groups (n = 8 in the control group, n = 10 in other groups).

#### Animal Model of HAPE

Rats were exposed to a simulated altitude of 6000 m (19,685 feet) in a hypobaric chamber (Institute of Aviation Medicine, Beijing, China) for 24 h. The rate of ascent to altitude was maintained at 300 m/min and it took 20 min to reach the desired altitude. After exposure for 24 h, the altitude descended to sea level at the same rate as the ascent to altitude. The temperature of the hypobaric chamber was maintained at 25 ± 1°C and the humidity was at 40-50%. The air flow rate was 4 L/h and the barometric pressure 355 mmHg. Rats were provided with adequate quantities of food and water during exposure to hypoxia.

#### HBO Exposure

Rats were placed into a custom-made pressure chamber of transparent acrylic plastic (701 Space Research Institute, Beijing, China) and received 1 h of HBO at 2.0 ATA in 100% oxygen and main-
and received 2 h of normobaric oxygen therapy. The chamber was flushed with 100% oxygen at a rate of 2 L/min to avoid accumulation of carbon dioxide and to maintain a stable oxygen concentration. The content of oxygen and carbon dioxide was continuously monitored and maintained at 35% and ≤ 0.03%, respectively. The temperature of the chamber was maintained between 22°C and 25°C and humidity at 40-50%.

**W/D Weight Ratios of Lung Tissue**

Rats were euthanized and thoroughly exsanguinated before the lung tissues were excised en bloc. Lung tissue samples were blot-dried and placed in pre-weighed trays made of aluminum foil. The wet weight of lung tissue was registered immediately on an electronic balance to an accuracy of 0.1 mg. The tray containing lung tissue was then baked in an oven at 70°C for 48 h until a constant weight was achieved. The dry weight of the tissue was then recorded. The water content of the tissue was calculated as wet weight minus dry weight, and expressed as milligrams of water per milligrams of dry tissue.

**Lung Morphology**

Lung tissue was fixed in 10% buffered formalin for 24 h. It was then embedded in paraffin and cut into 3 µm-thick sections. Sections were stained with hematoxylin and eosin, and images taken with an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a 40× objective lens.

In addition to W/D weight ratios of lung tissue, a published scoring system was applied to evaluate the lung injury visible under a light microscopy. The degree of injury was scored based on alveolar and interstitial edema, neutrophil infiltration, and hemorrhage. Injury severity was graded for each variable: no injury = 0; injury to 25% of the field = 1; injury to 50% of the field = 2; injury to 75% of the field = 3; and diffuse injury = 4. Samples were analyzed based on a scaled grading system by a pathologist who was blinded to the experimental protocol and the region of sampling. Three slides from each lung sample were randomly screened, and the mean taken as the representative value of the sample. The general injury score = edema score + neutrophil infiltration score + hemorrhage score. For presentation, we chose typical examples which were observed in all preparations for the same treatment.

**Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from frozen lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and a RNA kit (Bioer, Beijing, China). RNA was then reverse-transcribed to synthesize first-strand cDNA (Bioer, Hangzhou, China). Quantitative RT-PCR was done using a Line-Gen Sequence Detector (Bioer, Hangzhou, China). The primers of AQP1, AQP5, and beta-actin are listed in Table 1. PCR was performed using a BioEasy SYBR Green I Real-Time PCR Kit (BioMed). For the analysis of AQP1 and AQP5, the reaction mixture contained 25 µL of 2XSYBR Mix, 1 µL of 10 µM of forward primer, 1 µL of reverse primer, 0.3 µL of Taq DNA Polymerase, 2 µL of cDNA and 20.7 µL of ddH₂O. The thermal cycling conditions were: 95°C for 2 min for 1 cycle followed by 45 cycles of 95°C for 20 s, 60°C for 25 s, and 72°C for 30 s. Data was analyzed by the software attached to the detector (Bioer, Hangzhou, China). The size of RT-PCR products was confirmed by electrophoresis on 1% agarose gels. PCR products were sequenced, the sizes of amplicons for AQP1, AQP5 and beta-actin were 213 bp, 186 bp, and 150 bp, respectively. The relative quantification of mRNA expression was calculated by the 2^ΔΔCt method.

**Protein Preparation**

Peripheral lung tissues were frozen in liquid nitrogen, and stored at -80°C until analysis. Tissues were homogenized in ice-cold isolation solution containing 250 mM sucrose, 10 mM triethanolamine, 1 µg/mL leupeptin, and 0.1 mg/mL phenylmethyl sulphonylfluoride. Homogenates were centrifuged at 12000 rpm for 10 min at 4°C. Supernatants were obtained, and protein concentrations measured using a protein assay kit (Sunbio, Beijing, China). An N-glycosidase F deglycosylation kit (Roche, Mannheim, Germany) was used for deglycosylation of proteins.

**Western Blot**

Total proteins (50 µg/sample) were diluted in 5× loading buffer (Tris HCl 0.25 M, pH = 6.8; sodium dodecyl sulfate (SDS) 10%; bromophenol blue 0.5%; glycerol 50%; dithiothreitol 0.5 M), then boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% gradient gels. Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes treated with methanol. Membranes were blocked for 1 h at room temperature in TBS-T (Tris-buffered saline containing 0.1% Tween 20) containing 5% non-fat dry milk and incubated overnight at 4°C with anti-AQP1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-AQP5 antibody (1:200; Santa Cruz Biotechnology) in TBS-T containing 5% non-fat dry milk.

**Table 1. Sequences of Primers.**

| Gene | Primer (upstream) | Primer (downstream) |
|------|-------------------|---------------------|
| AQP1 | 5'-CTTGTCTGGTCCTTTGG-3' | 5'-ACCTTTCATGCCTTGTA-3' |
| AQP5 | 5'-ATCTCTGCTTCTTGGGCGT-3' | 5'-CAGCGAGATCTGTGTTGTA-3' |
| Actin | 5'-CTTGTCTGGTGGGCT-3' | 5'-TTT AAT GTC ACG CAC GAT TTC-3' |

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| Actin | 5'-CTTGTCTGGTGGGCT-3' | 5'-TTT AAT GTC ACG CAC GAT TTC-3' |
After washing in TBS-T, membranes were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit antibody (1:3000; Santa Cruz, Biotechnology) for 2-3 h at room temperature. Blots were developed with enhanced chemiluminescence agents (ECL Plus; Sunbio) before exposure to X-ray film. To confirm equivalent loading of samples, the same membranes were incubated with anti-β-actin antibody (1:1000; Santa Cruz Biotechnology) and visualized via enhanced chemiluminescence as described above. For quantification, films of western blots were scanned using a Minolta scanner and Adobe Photoshop software. The densitometry analysis was performed using LabWorks software (UVP, Upland, CA, USA). The relative density of AQP1 and AQP5 bands was normalized to the density of actin to represent the amount of AQP1 and AQP5 protein. The value of the control group was regarded to be 100%. The results of the HBO-PC and HAPE groups were expressed as a percentage of the value of the control group.

**Statistical Analyses**

Statistical analyses were carried out using SPSS15.0 software (SPSS, Chicago, IL, USA). All quantitative data were expressed as mean ± SD. One-way analysis of variance (ANOVA) or independent sample t-test were used to determine the differences among groups. P < 0.05 was considered significant.

**Results**

**W/D Weight Ratio**

The W/D ratio in the HAPE group was higher than that of the control group (p < 0.05), indicating increased lung water content in the HAPE group. However, no significant difference was observed between the HBO group and NBO group, or between the HBO group and NA group, suggesting no significant advantage of HBO over NBO and NA with respect to lung water content (Figure 1).

**Lung Morphology**

When the altitude was raised to 6000 m, rats demonstrated symptoms of lethargy, tachypnea and respiratory distress in the hypobaric chamber. However, after exposure for 24 h, with altitude descent, all above-mentioned symptoms were alleviated. When the chest was opened, the lungs of HAPE rats exhibited congestion, swelling, and hemorrhage. Histological examination of the lungs revealed marked interstitial edema in all sections in the HAPE group, suggesting that a rat model of HAPE was established. In this model, interstitial edema was described as a thickened interalveolar septum, expandable capillaries, and pink exudation in the alveolar space. The injury scores in the HBO group and NBO group were significantly decreased compared with the HAPE group (both p < 0.01). The injury score in the HBO group was significantly lower than that in the NA group (p < 0.01) and NBO group (p < 0.05). These results implied that oxygen exposure attenuated lung injury induced by hypobaric hypoxia at high altitude. Additionally, HBOT showed significant advantage over NBOT with respect to lung injury scores (Figure 2).

**The Protein Levels of AQP**

Western blot revealed that the expressions of AQP1 and AQP5 in the HAPE group were significantly reduced compared with the control group (both p < 0.01), implying that impaired movement of water resulting from the downregulation of AQP1 and AQP5 was correlated with the formation of HAPE. Significant differences were observed in the expressions of AQP1 and AQP5 between HBO and NBO, and the HBO and NA groups (both p < 0.01). AQP1 in the HBO and NBO group (but not in the NA group) was markedly increased compared with that in the HAPE (p < 0.01). These results indicated that the downregulation of AQP1 and AQP5 in the lungs of HAPE rats could be upregulated by oxygen exposure. HBO had more significant effect on the expressions of AQP1 and AQP5 than NBO and NA (Figure 3).

**The mRNA Levels of AQP1 and AQP5**

The mRNA transcription level of AQP1 in the HAPE group was significantly lower than that in the control group (p < 0.01). Significant differences were also found between the HBO and NBO, and the HBO and NA groups (p < 0.01). The mRNA level of AQP1 in the HBO and NBO groups were much higher than that in the HAPE (p < 0.01). These results indicated that the downregulation of AQP1 and AQP5 in the lungs of HAPE rats could be upregulated by oxygen exposure. HBO had more significant effect on the expressions of AQP1 and AQP5 than NBO and NA (Figure 3).
it was also significantly lower in the HAPE group than in the control group \( (p < 0.01) \). A significant difference was only found between the HBO and NA groups \( (p < 0.01) \), but not between the HBO and NBO groups. There were no significant differences between the HAPE and HBO, and between the HAPE and NBO groups. Furthermore, the mRNA level of AQP5 in the NA group was lower than that in the HAPE group \( (p < 0.05) \). Taken together, AQP1 and AQP5 play an important role in the pathogenesis of HAPE. Compared with NA, HBO had evident effect on the upregulation of AQP1 and AQP5 in lung tissues. Compared with NBO, HBO had more robust regulatory effect on the mRNA expression of AQP1 in the lung (Figure 4).

**Discussion**

**Establishment of the HAPE Model**

There are 2 types of pulmonary edema: interstitial pulmonary edema and alveolar pulmonary edema. If the fluid in
pulmonary interstitial edema cannot be absorbed effectively, alveolar pulmonary edema will develop. Therefore, interstitial pulmonary edema is an important pathological phase of alveolar pulmonary edema. Observations in mountain climbers revealed that nearly 80% of fast-ascending climbers had subclinical interstitial pulmonary edema, whereas the incidence of full-blown HAPE is much lower, suggesting that interstitial pulmonary edema is more common among mountaineers. In the current study, we investigated the effect of HBOT on the expressions of AQP1 and AQP5 in a rat model of interstitial pulmonary edema, in which rats were exposed to a simulated altitude of 6000 m for 24 h. Our results revealed that rats in the HAPE group exhibited typical interstitial pulmonary edema and demonstrated significantly increased lung water content, implying that the HAPE model in rats was successfully established.

HBOT and HAPE

The beneficial effects of HBOT on HAPE include increased tissue oxygenation, inhibition of ischemia–reperfusion injury, modification of neutrophil function, and impairment of bacterial replication, which are involved in theoverall antioxidant and anti-edema effect. HBO specifically suppresses tumor necrosis factor-alpha (TNF-α) and hypoxia-inducible facor-1 (HIF-1), which have been proven to be related to the pathogenesis of HAPE. The exposure to HBO enhanced K-ATPase activity, increased active Na\(^+\) transport, and accelerated lung edema clearance. All of these features may significantly improve the outcomes in HAPE. Liu et al. compared the therapeutic effect of HBOT plus conventional therapy with conventional treatment alone in 90 patients with HAPE. They found that HBOT, if used as an adjuvant, could shorten the duration of pulmonary edema. Duan et al. observed 32 HAPE patients from a plateau of 4636-5130 m and reported that, HBOT significantly reduced the elevated pulmonary arterial pressure in HAPE compared with NBOT. HBOT is highly recommended in treating HAPE among patients at extremely high altitude.

In the present study, oxygen exposure had more beneficial effect on HAPE than normal air exposure. Except for W/D ratio and mRNA expression of AQP5, HBO had significant advantage over NBO and NA, which was consistent with the above-mentioned studies. During the procedure of establishing HAPE models, rats gradually developed lethargy, tachypnea, and respiratory distress with the simulated increase in altitude in the hypobaric chamber. Also, the intake of food and water was reduced and some rats did not eat and drink during the exposure. The findings might result in a reduction in water content. After being taken out of the hypobaric chamber and treated with normal air, all these symptoms were improved, which accordingly led to varied degree of increase in water content.

The main concern in the use of HBO for pulmonary disorders is the fear of oxygen-induced pulmonary toxicity. A review showed that HBO-induced lung injury was related to prolonged treatment at high therapeutic pressure, and appeared to be less of a concern at hyperbaric pressures < 2.0 atmospheres. Our results indicated that the properly use of worked well on HAPE and had advantages over normobaric oxygen therapy.

AQPs and HAPE

The pathogenesis of HAPE is complicated. In general, the accepted mechanism is a sequential process of high-altitude hypoxia-induced pulmonary hypertension, increased capillary permeability, and compromise of the alveolar epithelial barrier resulting in pulmonary edema. In this process, injury to the capillary endothelium and alveolar epithelium appear to play
an important role in HAPE development. More than 90% of the internal surface area of the lung is lined by alveolar epithelial Type-1 cells, and AQP5 is mainly distributed in the apical membrane of Type-1 alveolar epithelial cells. AQP1 in primarily located in microvascular endothelia. Therefore, it is likely that the injury in the capillary endothelium and alveolar epithelium affect the function and expression of AQP1 and AQP5. Studies have indicated that AQP1 and AQP5 provide the principal route for osmotically-driven water transport across the microvascular endothelial barrier and alveolar epithelia, respectively. Considering the important role of AQP1 and AQP5 in ALI and formation of pulmonary edema, we hypothesize that water transport mediated by AQP1 and AQP5 may be involved in the pathogenesis of HAPE.

The expressions of AQP1 and AQP5 in the lungs of rats in the HAPE group were significantly decreased compared with the control group. Except for the mRNA level of AQP5, the expressions of AQP1 and AQP5 in the HBO and NBO groups were increased with the relief of symptoms. These data suggested the important roles of AQP1 and AQP5 in the pathogenesis of HAPE. In the lungs of HAPE rats, the lower expression of AQP1 and AQP5 may affect the absorption of water in the airspace, interstitial, and capillary compartments, which could consequently result in disordered fluid transportation and hypobaric hypoxia edema. Recent evidence suggested that AQP-1 transports nitric oxide (NO) across cell membranes.

The present study also demonstrated that the decrease in the level of AQP5 was less than that of AQP1. Because of the different distribution of AQP1 and AQP5 in the respiratory system, their functions on water transport in the lungs differ. AQP1 mainly affects interstitial edema and AQP5 affects alveolar edema. In the present study, most of the rats in the HAPE group had interstitial edema, which might explain why AQP1 expression decreased more than AQP5 expression.

Another interesting finding in the present study was that different from AQP1, the AQP5 protein, AQP1 mRNA, and AQP5 mRNA were decreased in the NBO and NA groups compared with the HAPE group, which may be due to the fact that the effect of hypobaric hypoxia at high altitude on the downregulation of AQP5 lasted for several hours. Jiao et al. obtained a similar result in a LPS-induced model of ALI in rats, in which AQP1 expression was partly resumed at 24 h after LPS treatment and steroid administration, whereas the expression of AQP5 was unchanged. In the present study, only the mRNA expression of AQP5 in the HBO group was increased compared with the HAPE group, suggesting a significant effect of HBO on the upregulation of AQP5 mRNA.

Studies on the regulation of AQPs showed that the expression of AQP is regulated by growth factors, TNF-α, inflammatory mediators, and osmotic stress. We wondered how HBO regulates the expression of AQP1 and AQP5 at the mRNA and protein levels. We hypothesized that HBO changes osmotic stress by improving the epithelial Na⁺ channel (ENaC) and the enzymatic activity of Na⁺-K⁺-ATP kinase, and HBO regulates HIF-1 and its downstream genes, including vascular endothelial growth factor (VEGF), TNF-α, and some inflammatory mediators.

There are some limitations of this study. First, the sample size was relatively small and only male rats were analyzed. Second, the varied food and water intake among groups might affect the W/D ratio of the lung tissues. Third, the regulation of AQPs on other signaling pathways or ion channels needs to be explored.

Conclusions

The present study demonstrated that HBO alleviated lung injury induced by high-altitude hypobaric hypoxia in rats. HBO showed significant advantage over NBOT. The pulmonary edema in rats induced by high-altitude hypoxia were associated with the downregulation of AQP1 and AQP5 in the lung, and this downregulation could be attenuated by HBO therapy. This is the first study showing the effect of HBO exposure on the mRNA and protein expressions of AQP1 and AQP5 in a rat model of HAPE. Further investigations are needed to identify the therapeutic role and the mechanisms of HBO on HAPE.

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Author Contribution

The conception or design of the work: Jiewen Tan, Chunjin Gao, Zhuo Li. The acquisition, analysis, or interpretation of data: Chunjin Gao, Cong Wang, Linlin Ma, Xiaomin Hou, Xuehua Liu. The creation of new software used in the work: Linlin Ma. Drafted the work and substantively revised the work: Xuehua Liu, Zhuo Li.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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