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Expression of aquaporin 1 and 4 in rats with acute hypoxic lung injury and its significance

Y. Wu[1], C.Y. Pan[2], C.Z. Guo[1], Z.J. Dong[1], Q. Wu[1], H.M. Dong[3] and W. Zhang[1]

[1]Department of Pathology, Medical College of QingHai University, XiNing, QingHai Province, China
[2]Department of Pathology, Nanyang Medical College, NanYang, HeNan Province, China
[3]Chinese People's Liberation Army Fourth Army Hospital, QingHai Province, China

Corresponding author: W. Zhang
E-mail: qhzhangwei_l@163.com

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ABSTRACT. Aquaporin (AQP)-1 and AQP-4 expression in lung tissues of SD rats during high altitude hypoxic lung injury, and the relationship between AQP-1 and AQP-4 expression, and acute hypoxic lung injury was analyzed. Thirty six healthy SD rats were divided into hypoxia 1d, 2d, 3d, 5d, and 7d groups and control group (N = 6). Pathological changes in lung tissue were observed by hematoxylin and eosin staining; lung injury was scored, and ultrastructural changes in lung tissue were observed by transmission electron microscopy. Changes in moisture content in lung tissues were determined by analyzing the wet/dry weight ratio (W/D). Localization of AQP-1 and AQP-4 was determined by immunohistochemistry. AQP-1 and AQP-4 expression were detected by western blot. Lung W/D was lower in hypoxia groups than in control group, and the highest in 3d group ($P < 0.05$). Light microscopy revealed a thickening alveolar wall and outstretched and congestive alveolar wall in hypoxia group; electron microscopy revealed

the presence of abnormal alveolar type II epithelial cells, cavitation in cytoplasm, microvillus-like protrusions, and a reduced lamellar body. AQP-1 and AQP-4 were mainly distributed in the capillaries and lymphatic and alveolar epithelial cells and airway epithelial cells, respectively. AQP-1 protein expression was decreased (western blot) in hypoxia 1d group (the lowest in 3d group; $P < 0.05$); there were no significant changes about AQP-4 expression. Therefore, AQP-1 may be involved in abnormal transport of liquid ALI and pathogenesis of lung edema. AQP-4 may not be involved in the formation of ALI lung edema.

Key words: High altitude hypoxia; Lung injury; AQP-1; AQP-4

INTRODUCTION

Acute lung injury (ALI) is a clinical syndrome characterized by highly permeable lung edema; so far, its pathogenesis has not been fully elucidated. Alveolar water transport was believed to be associated with the active transport of sodium, because of the lack of awareness of the function of aquaporins (AQPs) in lung tissues. AQPs comprise a group of cell membrane transport proteins that are involved in the transport of liquid under physiological conditions, as well as the pathological state of fluid transport. At present, studies on the function of AQPs in ALI are mainly focused on AQP-1 and AQP-5, with research on AQP-4 lacking. Research into whether AQP-1 and AQP-4 expression in the lung tissue is increased or decreased following ALI remains controversial. The changes in expression of AQP-1 and AQP-4 in lung tissues after acute hypoxia ALI have not been previously reported. In this study, we have investigated the changes in expression of AQP-1 and AQP-4 in lung tissues using a low-pressure oxygen cabin to simulate the ALI model of rats, and to elucidate its role in lung edema in ALI rats during the early hypoxic stage.

MATERIAL AND METHODS

Animals and reagents

Forty healthy adult SD rats (specific pathogen-free level) were purchased from the animal experiment center of Xi'an Jiao Tong University; the weight of these rats ranged from 150 to 250 g. The AQP-1, AQP-4, and GAPDH antibodies, goat-anti rabbit IgG, and DAB Color kit were purchased from WuHan Boster Biological Technology Co., Ltd., WuHan City, China.

Animal models and grouping

Thirty-six SD rats were purchased from Xi'an (altitude 400 m), their weights ranging from 210 to 230 g; these were randomly divided into 6 groups (N = 6). Rats in the control group were tested in the plains (altitude 400 m), the other 5 groups were quickly transported to the Qumalai county of Qinghai Province, with an altitude of 4500 m, within 10 h; the experiments were conducted in high altitude at 1, 2, 3, 5, and 7 days post-transport. The related indices were tested at the same time.

Determination of lung wet/dry ratio (W/D)

The middle left lung tissue of rats of each group was baked in an oven at 80°C for 24 h, until a constant weight was reached. The weights of the lung tissues were recorded before and after baking, and the W/D ratio was calculated.

Morphology of lung tissue

The left lung apex of rats was obtained and fixed with 4% paraformaldehyde; the tissues were cut into 4-μm thick slices and observed by hematoxylin and eosin (H&E staining after dehydration and embedding in paraffin. The morphology was scored using the Smith pathological lung injury score (Smith et al., 1997). The tissues were scored according to the four aspects of lung edema: alveolar and lung interstitial inflammation, alveolar and interstitial hemorrhage, and lung atelectasis. The scores ranged from 0 to 4 points: 0 indicated a normal lung, 1, 2, 3, and 4 indicated mild, moderate, severe, and extremely severe inflammation.

Ultrastructure of lung tissue

Tissues were obtained from the middle lobe of the right lungs of rats, fixed with glutaraldehyde, immersed in 0.1 M phosphate buffer solution, and again fixed with 1% osmium tetroxide. The samples were then dehydrated using an ethanol gradient; subsequently, the ethanol was replaced with propylene oxide, and the samples were embedded using epoxy resin 618. The ultrathin sections were stained with uranyl acetate lead citrate, and the ultrastructural changes in the lung tissues were observed under a transmission electron microscope.

Distributions of AQP-1 and AQP-4 in lung tissue observed by immunohistochemistry

The strept avidin-biotin complex (SABC) method was used to subject the slice to regular dewaxing and hydration; the endogenous enzymes were removed with 3% hydrogen peroxide and repaired with high-pressure antigen. The remaining steps were conducted according to the protocols provided by the manufacturers of the kit [SABC-POD(F) rabbit IgG kit, SA1028, Boster]. PBS was used as the negative control to instead of the primary antibody. AQP-1 and AQP-4 antibodies were diluted to 1:200. Intracellular brown coloring showed positive expression of AQP-1 and AQP-4 in the cells.

Expression of AQP-1 and AQP-4 in lung tissues detected via western blot

Forty milligram of the frozen tissue was taken and 200 μL protein lysate was added to every 10 mg of the tissue; a tissue homogenate was obtained using electric grinder at 4°C on ice. The homogenate was centrifuged at 12,000 *g* for 20 min. The supernatant was packed into a 1.5-mL centrifuge tube and preserved at -80°C. In strict accordance with the manufacturer instructions, the samples obtained were electrophoresed, transferred onto a film, and incubated with the primary antibody, secondary antibody, and the coloring agent in sequence. The color film was scanned and gray analysis performed using the ImageJ software; subsequently, the relative gray value was calculated.

Statistical analysis

All data are reported as means ± standard deviation; each group was subjected to a single factor analysis of variance using the SPSS v.17.0 statistical software (IBM, Armonk, NY, USA).

RESULTS

General situation

The rats in the hypoxic one-day (1d) group showed a good reaction and a ruddy complexion; the rats in hypoxic two-day (2d) group showed better reactions, a reduced diet intake and mental fatigue. The rats in the hypoxic three-day (3d) group were dispirited, appeared anorexic, and exhibited diarrhea, shortness of breath, nose and lip cyanosis, and irritability; rats in the hypoxia five-day (5d) group showed mild cyanosis and mental vibration; while the rats in the hypoxia seven-day (7d) group showed weight loss and anorexia, but no cyanosis.

Ratio of W/D in lung tissue

The W/D of lung tissues in the experiment group differed significantly from that of the control group; in addition, the W/D of the hypoxia 3d group also differed significantly from those of the other experimental groups ($P < 0.05$).

H&E staining of lung tissue

The nucleus was stained blue and cytoplasm stained pink under a light microscope. The lung tissues of rats in the normal control group showed a clear structure, with no lung interstitial edema and hyperemia in the capillary. The hypoxia 1d group exhibited alveolar cavity enlargement, alveolar wall thickening, interstitial lung capillary dilation, and congestion, and inflammatory cell infiltration. The rats in the hypoxia 2d group showed aggravated lung injury, while the hypoxia 3d group exhibited lung interstitial edema, continued alveolar wall thickening, reduced alveolar cavity, interstitial lung capillary dilation, reduced number of red blood cells in the alveoli, and increased infiltration of inflammatory cells. The lung injury of rats increased with the increase in time of hypoxia; the thickening of the alveolar wall was mitigated in rats of the hypoxia 5d group, and rats in the hypoxia 7d group exhibited infiltration of a few neutrophils in the alveoli and interstitial lung, with a continuous reduction in the thickening of the alveolar wall (Figure 1A-F). Determination of the pathological score of each group indicated that the scores of the hypoxia groups were higher than those of the control group ($P < 0.05$), with the hypoxia 3d group displaying the highest score ($P < 0.05$; Table 1).

Ultrastructure of lung tissue

The TEM ultrastructure of the lung tissues of rats from the hypoxia 1d group showed abnormalities in the alveolar type II epithelial cells, decreased microvilli, vacuolar cytoplasm, and decreased lamellar bodies. The hypoxia 2d group displayed swollen mitochondria in the cytoplasm of alveolar type II cells and slight hyperplasia in the alveolar mediastinum. We observed a reduction in the alveolar type II cells and partial lamellar bodies in the cytoplasm in the hypoxia 3d group. The ultrastructure of lung tissue in the hypoxia 5d group showed mild abnormalities in the alveolar type II cells, reduced membrane microvilli, dilation of cytoplasmic endoplasmic reticulum, and a small

quantity of lamellar structures; abnormalities in the alveolar type II cells and plasma membrane protrusions were considerably reduced in the hypoxia 7d group. However, the cytoplasm displayed a slightly more plate layer structure (Figure 2A-F).

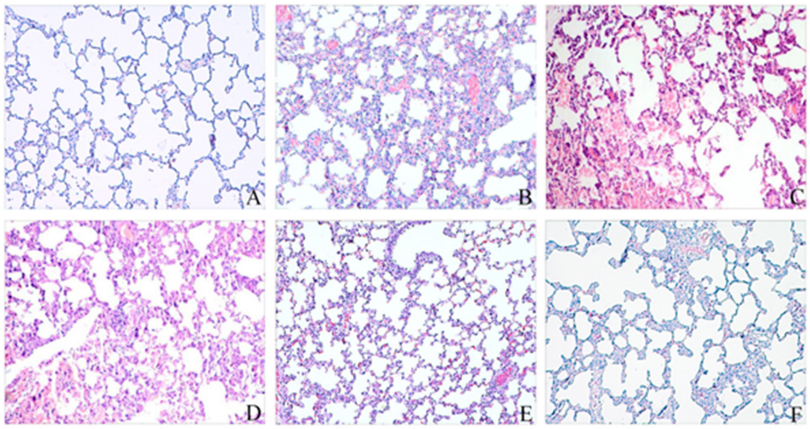

Figure 1. H&E staining of lung tissues in each group (100X). **A.** Normal lung tissues of the control group. **B.** Hypoxia 1d group. **C.** Hypoxia 2d group. **D.** Hypoxia 3d group. **E.** Hypoxia 5d group. **F.** Hypoxia 7d group.

Table 1. Indices of rats with hypoxic lung injury.

Group	N	W/D	Smith score	AQP-1 (Gray ratio with GAPDH)	AQP-4 (Gray ratio with GAPDH)
Control group	6	5.28 ± 0.60	0.34 ± 0.10	1.498 ± 0.026	1.032 ± 0.016
1 day	6	7.69 ± 0.33*	2.67 ± 0.52*	1.106 ± 0.027*	1.025 ± 0.043
2 day	6	9.11 ± 0.82*	6.81 ± 0.43*	0.665 ± 0.056*	1.028 ± 0.036
3 day	6	9.86 ± 1.38*$^{\Delta}$	7.50 ± 1.52*$^{\Delta}$	0.434 ± 0.038*$^{\Delta}$	1.037 ± 0.012
5 day	6	8.25 ± 1.01*	4.93 ± 1.07*	0.602 ± 0.074*	1.026 ± 0.060
7 day	6	7.12 ± 0.21*	2.00 ± 0.89*	1.305 ± 0.015*	1.019 ± 0.027

*Compared to the normal control group, $P < 0.05$; $^{\Delta}$Comparison with each experimental group, $P < 0.05$.

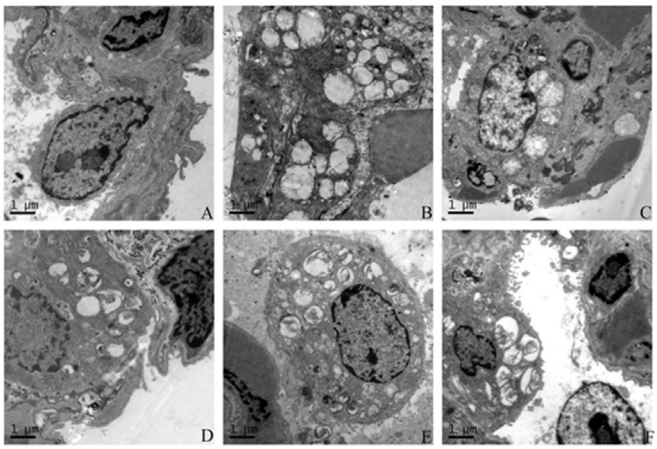

Figure 2. Ultrastructure of a lung tissue (10,000X). **A.** Normal lung tissue of the control group. **B.** Hypoxia 1d group. **C.** hypoxia 2d group. **D.** Hypoxia 3d group. **E.** Hypoxia 5d group. **F.** Hypoxia 7d group.

Changes in AQP1 and AQP4 expression in lung tissues observed by immunohistochemistry

We observed a high level of expression of AQP-1 in the lung tissues of the control group, and an obvious yellow-to-brown distribution in the lung capillaries and alveolar endothelial cells. Compared to the control group, the simulation plateau hypoxia groups showed decreased pigmentation indicating the presence of the AQP-1 protein in the lung tissues. AQP-4 was mainly distributed in the airway epithelial cells; we observed no significant changes in the distribution and expression of AQP-4 during lung injury compared to the control group (Figures 3A-F and 4A-F).

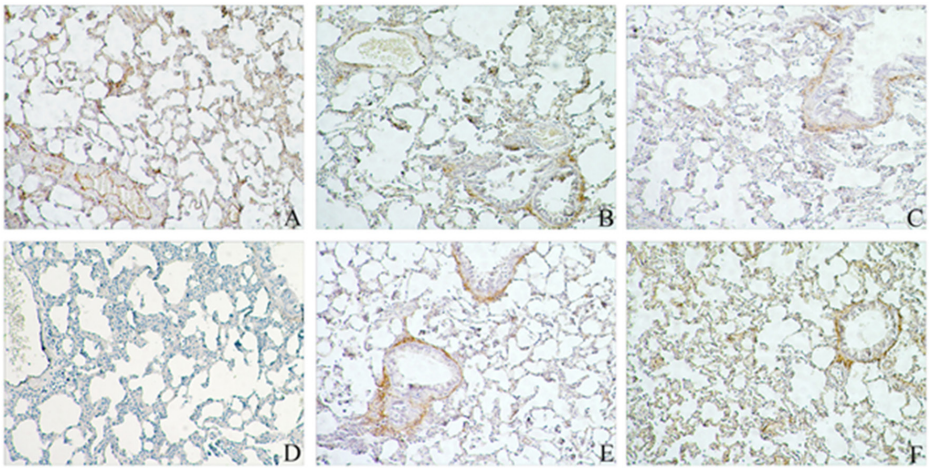

Figure 3. Expression of AQP-1 in the lung tissue of each hypoxia group (SABC 200X). **A.** Normal lung tissue of the control group. **B.** Hypoxia 1d group. **C.** Hypoxia 2d group. **D.** Hypoxia 3d group. **E.** hypoxia 5d group. **F.** Hypoxia 7d group.

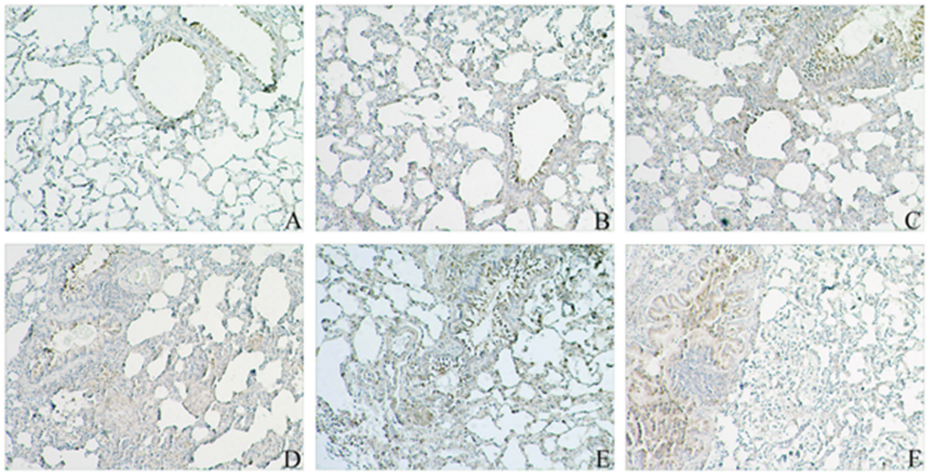

Figure 4. Expression of AQP-4 in the lung tissues of each hypoxia group (SABC 200X). **A.** Normal lung tissue of the control group. **B.** Hypoxia 1d group. **C.** Hypoxia 2d group. **D.** Hypoxia 3d group. **E.** hypoxia 5d group. **F.** Hypoxia 7d group.

Changes in AQP1 and AQP4 expression in the lung tissue of each group, analyzed by western blot

The expression of AQP1 in the lung tissues of hypoxic rats was decreased compared to the normal control group. AQP-1 expression reached a peak value in the hypoxic 3d group; this difference was statistically significant. We observed no significant changes in the expression of AQP4 in any of the rats ($P < 0.05$; Table 1, Figures 5 and 6).

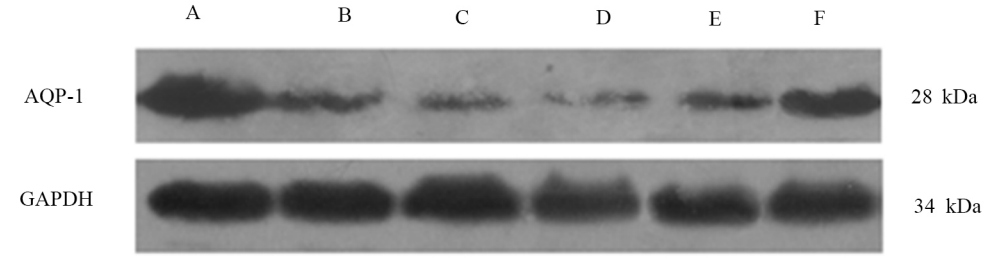

Figure 5. Western blot results of AQP-1 in rat lung tissues. **A.** Normal lung tissue of the control group. **B.** hypoxia 1d group. **C.** Hypoxia 2d group. **D.** Hypoxia 3d group. **E.** Hypoxia 5d group. **F.** Hypoxia 7d group.

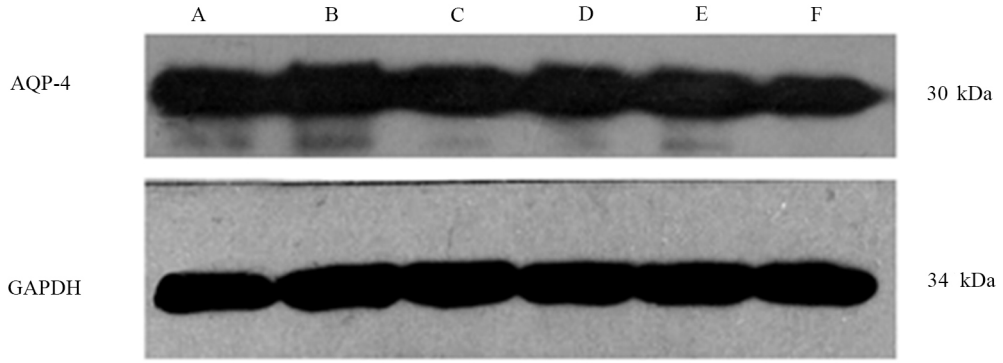

Figure 6. Western blot results of AQP-4 in rat lung tissues. **A.** Normal lung tissue of the control group. **B.** Hypoxia 1d group. **C.** Hypoxia 2d group. **D.** Hypoxia 3d group. **E.** Hypoxia 5d group. **F.** Hypoxia 7d group.

DISCUSSION

Hypoxia increases the vascular permeability of the lung and destroys the fluid balance in, and water barrier of lung tissues, which leads to severe ALI (lung edema). The pathological characteristics of lung edema include fluid accumulation in the alveolar and interstitial lung. Many research studies have shown that AQP is involved in the pathogenesis of lung edema (Singha et al., 2013). At present, 13 types of AQPs have been found in mammals (AQP 0-12), 6 of which are distributed in the lung tissues (AQP-1, -3, -4, -5, -8, and -9) (Zhu et al., 2008). Bai et al. (1999) also found a 10-fold decrease in the water permeability of the alveolar capillary membrane barrier following an *AQP*-1 gene knockout. Meanwhile, the water permeability was decreased 14 to 16 times after AQP-1 and AQP-4 knockout in mice. This indicated that AQPs selectively

allowed the passage of water molecules and mediated the transmembrane transport of free water molecules; therefore, these proteins are believed to closely influence the lung water balance and the occurrence of lung edema. However, the changes in AQP expression in the lung tissues as a result of ALI still need to be properly quantified. A majority of the studies showed a decrease in AQP expression (Towne et al., 2000); however, Lai et al. (2003) reported that inflammatory cytokines promote the expression of AQP-1 in a cell model. The expression of AQP-1 decreased significantly after subjecting them to 4-12 h of acute lung injury induced by acute pancreatitis. AQP-1 expression increased gradually after 12 h (Liu et al., 2014). Zhu et al. (2008), on the other hand, observed an increase in AQP-4 expression in rats with oleic acid-induced acute lung injury. Conversely, Li et al. (2008) reported that AQP-4 was not involved in the formation of ALI lung edema. These changes could possibly be attributed to the altered expression of AQP-1 and AQP-4 during the early stages of high altitude hypoxia, resulting in abnormal transmembrane transport and accumulation of water in the lungs, which in turn damaged the lung tissue. At present, this has not been reported in literature. In this study, a high altitude hypoxia lung injury animal model was simulated in SD rats, and the AQP-1 and AQP-4 expression in lung tissues were analyzed over a long period of time in the context of radical plateau hypoxia; we also investigated the relationship between hypoxic ALI and AQP-1 and -4.

The experimental results showed an increase in the lung W/D ratio in the hypoxic 1d group; the hypoxic 3d group showed the highest W/D, which decreased subsequently. This suggested a gradual increase in the lung fluid leakage during the acute phase of lung injury, and a high amount of exudate on day 3. Light microscopy revealed the thickening of the alveolar wall in experimental rats; in addition, the capillary was enlarged and filled with blood, and there was increased infiltration of red blood cells and macrophages in the alveoli and interstitial cells. Therefore, the pathological score of the lung tissue of rats in the experimental group was higher than that of rats in the control group ($P < 0.05$); the hypoxic 3d group showed the highest pathological score ($P < 0.05$). Different degrees of abnormal alveolar type II epithelial cells were seen in the experimental groups under an electron microscope; in addition, we observed swelling of the mitochondria in the cytoplasm, a decrease in microvilli, cavitation, and reduction in/emptying of the plate layer. The injuries were most obvious in the hypoxia 3d group, and the degree of the injury degree was mitigated in the 7d group. This suggested that the hypoxic environment might have caused ALI in rats. Immunohistochemistry revealed that AQP-1 was mainly expressed in the endothelial cells of capillary, around the bronchi and alveolar type II epithelial cells, while AQP-4 was mainly expressed in the airway epithelial cells. Results of the western blot analysis indicated a decrease in the expression of AQP-1 in hypoxia 1d rats, with the lowest level in 3d rats; AQP-1 expression increased gradually after 3 days ($P < 0.05$). AQP-1 is distributed in the peripheral lung vascular endothelium and bronchus, and is responsible for eliminating water from the bronchi and around the vessels. The levels of AQP-1 expression and the severity of lung edema are significantly related, suggesting an important association between AQP-1 and the formation of lung interstitial edema (resulting from dysfunction of the water clearance around the bronchi and vessels). We speculated that some inflammatory mediators might have damaged the endothelial cells during the early stage of hypoxic lung injury, or that alveolar type II epithelial cell apoptosis decreased the expression of AQP-1 in rat lung tissues, resulting in a decrease in the alveolar capillary water permeability and the scavenging capacity of lung tissues. This could in turn affect the absorption of edema fluid and aggravate the alveolar and interstitial edema, leading to the formation of lung edema. We observed no changes in the expression of AQP-4 in any of the group; this was attributed to the distribution

of AQP-4 in the airway epithelial cells. The main function of AQP-4 is to remove moisture from the airway; however, as lung injuries are mainly caused by lesions in the alveolar wall, with minimal damage to the airway epithelial cells, the expression of AQP-4 remained chiefly unaltered.

This study showed that AQP-1 plays a regulatory role in the formation of lung interstitial edema and the transport and clearance of water during the different stages of acute hypoxic lung injury in a rat model. The AQP-4 expression showed no obvious changes, indicating that it may not participate in the development of lung edema after ALI. The role of AQPs in the occurrence and development of lung damage must be further researched, in order to develop methods to improve the content or activity of AQPs and increase the water clearance of lungs in patients to treat acute altitude-related hypoxic lung injury.

Conflicts of interest

The authors declare no conflict of interest.

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