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Stress failure plays an major role in development of High Altitude Pulmonary Edema in rats

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ABSTRACT

Hypoxia and exertion are considered as the two main factors in the development of High altitude pulmonary edema (HAPE) but its pathophysiology remains unclear. Therefore, we established a model in which 32 Sprague-Dawley rats were randomly assigned to normoxic rest, hypoxic rest, normoxic exercise and hypoxic exercise.

An altitude of 4700m was simulated using hypobaric hypoxia, while exercise consisted in a 48 hours walk with 15-20min breaks every 4 hours. Arterial blood gas,broncholalveolar lavage (BAL), lung wet-to-dry weight ratio (W/D) and histological measurements were conducted on each animal.

In rats exercising in hypoxia, BAL protein and lung W/D ratio were significantly increased but no changes in BAL leukotriene B4 and IgM were observed. In the same group, Lung histology showed typical hemorrhagic lung edema and disruption of both alveolar epithelium and capillary endothelium while hypoxia or exertion alone only induced slight endothelium and epithelium swelling/disruption.

Our study established a direct link between histological and physiological evidence of HAPE-like symptoms and we demonstrated that hypoxia and exertion can synergistically induce HAPE-like symptoms in Sprague-Dawley rats without inducing lung inflammation. We therefore propose that alveolar epithelium and capillary endothelium stress failure play a major role in the development of HAPE

Key words: exercise; hypobaric hypoxia; high altitude pulmonary edema; rat model; stress failure

INTRODUCTION

High altitude pulmonary edema (HAPE) is a potentially fatal condition that may affect non-acclimatized individuals who ascend rapidly to altitude above 3000 m [1, 2]. Approximately 2% of individuals exposed to high altitude were affected severely enough by HAPE to seek treatment [3]. However, the incidence of sub-clinical HAPE may be as high as 75% in individuals traveling at an altitude above 4500 m [4]. Hypoxia and exertion have been identified as the two main factors in HAPE development [5]. However, the exact mechanisms that trigger the development of HAPE are not completely understood. Although factors such as inflammation and decreased alveolar fluid clearance are thought to be important in HAPE pathophysiology [6□7□8□9], pulmonary capillary stress failure, which results from an uneven hypoxic pulmonary vascoconstriction [10, 11] has been suggested to be the primary inciting mechanism. Indeed, increased pulmonary capillary transmural pressure [12, 13, 14] causes the rupture of the alveolocapillary membrane [15], allowing the flooding of the alveolar space with a protein rich and hemorrhagic edema fluid. Hopkins et al [16] found evidence of stress failure in exercising humans under hypoxic conditions,, and Swenson et al [17] showed the presence of red blood cell as well as increased protein concentration in BAL fluid form humans with HAPE, without significant changes in BAL cytokines. However, whether pulmonary capillary stress failure is the primary mechanism in the development of HAPE is not conclusive since direct evidence of capillary rupture cannot be obtained from humans

Therefore, an animal model is required to obtain direct histological evidence of capillary rupture in the lung. To date, rabbit, sheep, dogs and ferrets have been used to develop HAPE animal models but the results were neither consistent nor reproducible [18, 19, 20]. Rats have also been used in a number of studies, but either required endotoxin priming [19], exposure to severe hypoxia (236 Torr, 9000 m) or the use of specific rat strains such as the Madison strain which has a high susceptibility to the induction of HAPE [15, 21]. Some studies on pigs showed findings consistent with onset of HAPE when they were exposed to normobaric hypoxia [22], but no convincing histological evidence was provided.

In this study, we hypothesized that the combination of hypoxia and exercise would cause stress failure of the pulmonary capillaries and induce HAPE-like symptoms. In order to test this hypothesis, we established a HAPE model using rats exercising under hypoxic conditions. This model allowed us, for the first time, to directly link histological evidence for capillary breaks to BAL findings and wet-dry ratios.

2. MATERIALS AND MEDTHODS

2.1 Animals

Adult male Sprague-Dawley rats (Animal Center of Fudan University, Shanghai, China) weighting 252-298 g were used in this study. Rats were exposed to a 12 hours day-night light cycle for 2 days before starting experiments. All animal protocols were approved by the Animal Care Committees of Fudan University. All animal experiments

were conducted in accordance with the Guidelines for the Use and Care of Research Animals published by the National Institutes of Health.

2.2 Experimental design

Hypoxia setting:

A hypobaric hypoxia chamber (Vacuum Chamber, South China Shipbuilding Yard, Shanghai, China) was used to generate specific hypoxic conditions. Briefly, after closing the chamber, air pressure inside the chamber was progressively decreased by a vacuum connected to the chamber. The rate of pressure decrease was set to 20m/s until it reached a pressure equivalent to an altitude of 4700m (≈ 419.97 Torr).

Rats exercise using Treadmill:

Rats were trained to walk on a treadmill (DSPT-202, Duan Co., Ltd, Hangzhou, China) at a speed of 12m/min for 2 days before exposure to hypoxia. When the rats were exposed to hypobaric hypoxia, they were forced, using electrical stimulations, to walk on a treadmill at roughly 12m/min [23].

Experimental and control groups:

There were 8 rats in each group and the groups were as follow: rats exposed to normobaric normoxia at rest (**NR**); rats exposed to normobaric normaxia with exercise (**NE**); rats exposed to hypobaric hypoxia with exercise (**HE**); and rats exposed to hypobaric hypoxia at rest (**HR**). In the exercise groups (in either hypoxia or normoxia), the rats walked for 48 hours but were allowed to stop for a 15-20min every 4 hours, so they could have access to food or water. It is important to note that the rats were, however, continuously exposed to hypoxia during 48 hours.All procedures were

performed in the main chamber that investigator could access through an independently controlled antechamber where the pressure was set at 470.48 Torr (≈3800 m) [21]. When pressure inside of the main chamber decreased to 3800m, the investigator could enter into the main chamber to check the rats. Once the investigator intervention was completed, the chamber was set back at a pressure of 419.97 Torr (≈4700m). Thus, the rats were exposed to a simulated altitude of 4700 m for most of the time with brief exposures to 3800 m.

2.3 Arterial blood gases

The following procedure was performed in the main chamber with pressure equivalent to 3800 m for investigators safety [21]. After anesthesia (intraperitoneal injection of 10% chloral hydrate, 3mg/kg, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China**)**, blood was quickly collected by direct puncture and sampling from the left ventricle using heparinized syringes. All blood samples for arterial blood gas analysis were immediately analyzed by GEM Premier 3000 (Instrumentation Laboratory, Massachusetts, USA). Arterial blood pH, partial pressure of carbon dioxide ($PaCO_2$) and partial pressure of oxygen (PaO_2) were corrected according to the rectal temperature [22].

2.4 Lung wet-to-dry weight ratio (W/D)

The rat was placed in a supine position and a cannula was inserted into the trachea after tracheotomy. The lungs were isolated after chest opening, and the right superior lobe was cut to measure wet weight and then dried in an oven for 72 hours in order to get the

dry weight. Lung wet-to-dry weight ratio was calculated as described previously [24, 25].

2.5 Lung histology

The lungs were inflated with air at pressure of 20cmH_2O initially then held at 5 cmH_2O. After cutting the left atrium, the lungs were perfused with saline via the right ventricle at a pressure of 20-30 cmH_2O. When the outflow was clear of blood cells, the lungs were perfused for additional 10 minutes and stored in 4 % paraformaldehyde for light microscopy or 3 % glutaraldehyde for electron microscopy at 4□ [15]. After 5 days, lungs were embedded and sectioned. Hematoxylin and eosin stained sections were prepared using standard procedure [24]. The Microscopic lung injury was scored based on alveolar edema, interstitial edema, hemorrhage, neutrophil and macrophage infiltration. The grades for severity of lung injury in hematoxylin and eosin stained slices were as follows: no injury = 0; 25% of the field injured =1; 50% of the field injured = 2; 75% of the field injured = 3; and diffuse injury = 4 [26]. All histology samples were scored by two independent pathologists following a double blind protocol as done previously [24]. The electron microscopy imaging was performed as described previously [15]. A grading score was set in order to quantify endothelium/epithelium stress failure. This score consisted in the number of breaks observed per surface unit of examined sections.

2.6 Bronchoalveolar lavage (BAL)

After right lungs were ligated and sectioned, 1 ml 0.9% saline was injected into the left lung through left main bronchus. BAL fluid was withdrawn after 2-3 times gentle and

repeated flushing. A total of 3 ml were injected and BAL fluid was stored in a sterile container, filtered through sterile gauze to remove mucus and cell debris, and then kept at -80°C for later cytokines measurement [22]. The red and white blood cells were counted using Sysmex KX-21 (Sysmex Co., Ltd, Japan). Total protein and albumin were measured using by an Automatic Analyzer (HITACHI-7600, Hitachi Ltd, Japan). BAL Immunglobulin M (IgM) was measured by immunoturbidimetry using an Automatic Analyzer (HITACHI-7600, Hitachi Ltd, Japan) [27]. BAL LTB4 was detected by a rat ELISA kit (RapidBio Lab, Calabasas, California, USA) according to manufacture's protocol**.**

2.7 Statistical analysis

Data were presented as mean ± SEM and analyzed using SAS 6.12 software (SAS Institute Inc., North Carolina, USA). Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) procedures with Bonferroni test [24, 28]. The lung injury scores of histology were analyzed by the non-parametric Mann-Whitney test. $P< 0.05$ was considered statistically significant between groups. Pearson test was performed to evaluate data correlation. R is the correlation coefficient ($-1< R <1$). The statistical comparisons were always performed with the control group (NR) unless specified.

3. RESULTS

Three out of eight rats exposed to hypobaric hypoxia with exercise died between 27 and 35.5 hours. All animals in other three groups survived.

3.1 Arterial blood gases

We observed a significant decrease of PaO_2 in HE and HR rats compared to NR and NE rats (**Table 1**; $p < 0.01$). $PaCO_2$ in HE and HR rats were also reduced due to hyperventilation (**Table 1**; $p < 0.01$ and 0.05, respectively). A significant increase of pH was only observed for the HR rats (**Table 1**; $p < 0.05$). Based on arterial blood gases, we calculated alveolar-arterial differences for the different groups (**Table 1)**. The alveolar-arterial difference was calculated as follow:

$PA\text{-}aO_2 = PAO_2\text{-}PaO_2$ (where $PAO_2=(BP\text{-}PH_2O)*FIO_2\text{-}PaCO_2/R$; PAO_2 is the partial pressure of oxygen in the Alveolus; BP is the barometric pressure; PH_2O is saturated vapor pressure of water at 1 atmosphere and 37°C; FIO_2 is the fraction of inspired oxygen; $PaCO_2$ is the partial pressure of arterial carbon dioxide ≈ $PACO_2$; R is the respiratory quotient and was assumed at 0.8 in the above equation). These results showed a significant increase in the alveolar-arterial difference of HE group compared to NR, NE and HR groups, which suggests a decreased efficiency of pulmonary gas exchange.

3.2 Lung wet-to-dry weight ratio

Lung W/D ratio is an indicator of overall lung edema severity [29] The lung W/D weight ratio in HE and HR rats were significantly higher when compared to control (NR) rats (**Table 1**; $p < 0.01$ and 0.05, respectively). The W/D ratio of HE group was

significantly higher than NE group ($p < 0.01$). There was no significant difference of lung wet-to-dry weight ratio between NE and NR groups..

3.3 Bronchoalveolar lavage

BAL protein is an indicator of changes in lung alveolar-capillary membrane permeability [30]. The total protein concentration of BAL was increased significantly in HE and HR rats compared to NE and control NR rats (**Table 1**; $p < 0.01$). The albumin concentration in BAL from HE and HR rats was also significantly increased compared to NE and NR rats (**Table 1**; $p < 0.01$ and 0.05, respectively). Although the total protein and albumin in HE and HR rats were both increased, there was no significant difference between these two groups. Red and white blood cell counts in BAL from HE and HR rats were significantly increased compared to NE and NR rats (**Table 1**; $p < 0.01$ and 0.05, respectively). Red blood cell counts in HE rats were, however, significantly higher than in HR rats ($p < 0.05$). Based on Schoene et al. (1998) who observed increased levels of BAL IgM and LTB4 in human subjects, we performed BAL IgM and LTB4 measurements. There were no significant differences between the four groups (**Table 1**). BAL LTB4 data suggested that inflammation might not be involved in early stage of our HAPE model. However, BAL protein measurements as well as cell count suggested that hypoxia exposure increased alveolar-capillary permeability, while exercise synergistically amplified the barrier damage caused by hypoxia.

3.4 Gross appearance and light microscopy

Hemorrhagic fluid was noticed in the nose and mouth in three dead rats from HE group, indicating a severe pulmonary edema. Three out of five HE survivors and one out of the eight HR rats showed various degrees of lung hemorrhages. Compared with the NR rats (**Figure 1A**), there were markedly increased alveolar edema fluid, swollen/thickened pulmonary interstitium and red blood cells in the alveoli of HE non-survivors (**Figure 1D-1**) and HE survivors rats (**Figure 1D-2**). Lung histology in HR rats showed significantly thickened alveolar septum and few red blood cells in alveolar space, but no obvious alveolar edema (**Figure 1C**), suggesting that hypoxia *per se* does not induce HAPE-like symptoms. In NE rats, there was only a slight increase of red blood cells in interstitium, but no significant increase of alveolar and interstitial edema (**Figure 1B**). A summary of histological findings is provided in figure 2. Alveolar edema score in HE group was significantly increased compared to the other three groups (**Figure 2A**). Interstitial edema score in both HE and HR group were significantly increased compared to NR group (**Figure 2B**; $p < 0.01$). The hemorrhage score in HE was significantly increased compared to NE and NR groups (**Figure 2C**; $p < 0.05$ and 0.01, respectively). The neutrophil and macrophage infiltration increased in HE group compared to control (NR) group (**Figure 2D**; $p < 0.05$). In summary, morphological analysis showed that combination of hypoxia and exercise induced more severe alveolar-capillary barrier disruption when compared to hypoxia and exercise alone. This severe barrier disruption observed in HE rats was characterized by typical alveolar hemorrhage and edema.

3.5 Electron microscopy

Electron microscopy was used to show detailed ultra structure changes. An example of normal (from NR group) epithelial and endothelial barrier is shown in figure 6A. In HE rats, there was a complete rupture of blood-gas barrier (**Figure 3D-1**), including disruption of endothelial layer (**Figure 3D-2**), swelling of epithelium (**Figure 3D-3**) and disruption of epithelium (**Figure 3D-4**) compared to control rats (**Figure 3A**) and NE rats (**Figure 3B**). The epithelium was heterogeneously affected with either evidences of swelling or rupture (**Figure 3D-3 & D-4**) and red blood cell in the alveoli suggested the disruption and denudation of alveolar epithelium cells that favored alveolar flooding. In HR rats, slight endothelium and epithelium swelling as well as few red blood cells in the alveolar spaces suggested a situation similar to sub-clinical HAPE in humans (**Figure 3C**). Electron microscopy injury score showed that hypoxia or exercise alone only slightly increased endothelial and epithelial cell swelling/disruption, while hypoxia together with exercise significantly induced endothelial and epithelial cell disruption (**Figure 3E**).

4. DISCUSSION

Hypoxia and exertion are considered as the two main factors in development of HAPE, we hypothesized that exercising rats exposed to hypobaric hypoxia will develop HAPE-like symptoms. Therefore, we established a rat animal model to study the mechanisms of HAPE development due to exercise under hypoxic conditions.

Our results demonstrated that rats exercising in hypoxia developed typical HAPE-like symptoms as determined by both physiological and histological analysis such as increased alveolar-arterial difference (Table 1), increased W/D ratio (Table 1), increased BAL total protein/albumin content (Table 1), lung endothelial and epithelial cell swelling/disruption and alveolar flooding/hemorrhage (Figure 1, 2&3). To our knowledge, this study is the first to show that hypoxia and exercise synergistically induces HAPE-like symptoms in Sprague-Dawley rats. Indeed, although BAL total protein and albumin concentration were significantly increased in rats exposed to either hypoxia or exercise, the increase was higher when rats were simultaneously exposed to both hypoxia and exercise (Table 1), suggesting an exacerbation of endothelial/epithelial permeability [31, 32, 33]. Also, detailed electron microscopy analysis showed that hypoxia or exercise alone only induces slight alveolar epithelium and capillary endothelium swelling/disruption, while combined hypoxia and exercise significantly increased endothelium/epithelium swelling and disruption. This destruction of the alveolocapilllary membrane by the combination of hypoxia and exertion is underscored by the higher red blood cell count in the BAL from HE rats. In our model, HR rats present intermediate features/symptoms that could correspond to sublinical HAPE in humans. Indeed, the interstitial edema score, the BAL albumin concentration, the BAL red blood cell content and the W/D ratio are elevated compared to NR and NE groups but these parameters remain lower than that in the HE group and the alveolo-arterial difference ($PA\text{-}aO_2$) does not indicate an alteration of pulmonary gas exchanges.

Using this model, we also showed for the first time, a direct link between histological evidences for stress failure and other measurements such as wet-dry-ratio, BAL analysis and arterial blood gases. Indeed, capillary breaks positively correlated with edema score, W/D ratio, BAL albumin concentration and the alveolar-arterial difference (**Table 2**). These clear correlations strongly suggest that stress failure plays a major role in the development of HAPE-like symptoms in our model.

Another factor that has been proposed to play a role in the pathogenesis of HAPE is inflammation [6]. Thus we measured IgM and LTB4 in the BAL. However, no significant difference appears among the four groups for both LTB4 and IgM. The observation of distal lung lesions with no evidence of inflammation suggests that inflammation plays a minor role in the development of HAPE-like symptoms in our animal model [17]. Moreover, although the neutrophile & macrophage infiltration score was higher in the HE group, it was also elevated in the other experimental groups (HR and NE rats) and did not significantly correlate with either the edema score, the capillary breaks or the W/D ratio (**Table 2**).

Decreased alveolar fluid clearance (AFC) has also been proposed to contribute to the development of HAPE [7, 8, 9]. Indeed, in case of alveolar flooding, the alveolar fluid clearance constitutes the first line of defense by allowing alveolar fluid removal, a mechanism known to depend on active vectorial ion transport through the epithelium. Due to hypoxia mediated inhibition of function/expression of the alveolar epithelial sodium channel (ENaC) [7, 9], AFC will likely be affected in our model. However, regarding the lung lesions observed in the HE group, we do not think that decreased

AFC triggers the formation of HAPE in our model. AFC is currently under investigation in our animal model.

Our model does, however, have some limitations. Indeed, we could not measure pulmonary artery pressure due to measurement complexity in our experimental setting and the exercise intensity may not transpose to exertion levels observed in humans. We therefore propose that exacerbation of hypoxic pulmonary vasoconstriction by exertion would induce stress failure of the distal lung structure, promoting alveolar flooding with a protein rich and hemorrhagic edema.

In conclusion, **1.** Our model of exercising rats exposed to hypobaric hypoxia is able to induce HAPE-like symptoms without endotoxin priming, severe hypoxia or the use of special strain. **2.** Our HAPE model is not associated with a lung inflammation **3.** The correlation between elevated albumin concentration in the BAL as well as higher W/D ratio and increased endothelium/epithelium swelling/disruption suggested that alveolocapillary barrier stress failure plays a major role in the development of HAPE.

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REFERENCES

1. West JB. The physiologic basis of high-altitude diseases. *Ann Intern Med* 2004; 141: 789-800.

2. Mortimer H, Patel S, Peacock AJ. The genetic basis of high-altitude pulmonary oedema. *Pharmacology & Therapeutics* 2004; 101: 183-192.

3. Hackett PH, Rennie D. The incidence, importance, and prophylaxis of acute mountain sickness. *Lancet* 1976; 2: 1149-1155.

4. Cremona G, Asnaghi R, Baderna P, et al. Pulmonary extravascular fluid accumulation in recreational climber: a prospective study. *Lancet* 2002; 359: 303-309.

5. Basnyat B, Murdoch DR. High altitude illness. *Lancet* 2003; 361: 1967-1974.

6. Schoene RB, Swenson ER, Pizzo CJ, et al. The lung at high altitude: bronchoalveolar lavage in acute mountain sickness and pulmonary edema. *J Appl Physiol* 1988; 64: 2605-2613.

7. Sartori C, Allemann Y, Duplain H, et al. Salmeterol for the prevention of high altitude pulmonary edema. *N Engl J Med* 2002; 346: 1631-1636.

8. Scherrer U, Sartori C, Lepori M, et al. High-altitude pulmonary edema: from exaggerated pulmonary hypertension to a defect in transepithelial sodium transport. *Adv Exp Med Biol* 1999; 474: 93-107.

9. Zhou G, Dada LA, Sznajder JL. Regulation of alveolar epithelial function by hypoxia. *Eur Respir J* 2008; 31: 1107-1013.

10. Grunig E, Mereles D, Hildebrandt W, et al. Stress Doppler echocardiography for identification of susceptibility to high altitude pulmonary edema. *J Am Coll Cardiol* 2000; 35: 980-987.

11. Dehnert C, Grunig E, Mereles D, et al. Identification of individuals susceptible to high-altitude pulmonary oedema at low altitude. *Eur Respir J* 2005; 25: 545-551.

12. Maggiorini M, Melot C, Pierre S, et al. High-altitude pulmonary edema is initially caused by an increase in capillary pressure. *Circulation* 2001; 103: 2078-2083.

13. West JB, Mathieu-Costello O, Prediletto R. Stress failure in pulmonary capillaries. *J Appl Physiol* 1991; 70: 1731-1742.

14. Tsukimoto K, Mathieu-Costello O, Prediletto R, et al. Ultrastructural appearances of pulmonary capillaries at high transmural pressure. *J Appl Physiol* 1991; 71: 573-582.

15. West JB, Colice GL, Lee Y-J, et al. Pathogenesis of high-altitude pulmonary oedema: direct evidence of stress failure of pulmonary capillaries. *Eur Respir J* 1995; 8: 523-529.

16. Hopkins S, Garg J, Bolar DS, et al. Pulmonary blood flow heterogeneity during hypoxia and high-altitude pulmonary edema. *Am J Respir Crit Care Med* 2005; 171: 83-87.

17. Swenson ER, Maggiorini M, Mongovin S, et al. Pathogenesis of high-altitude pulmonary edema: inflammation is not an etiologic factor. *JAMA* 2002; 287: 2228-2235.

18. Schoene RB, Goldberg S. The quest for an animal model of high altitude pulmonary edema. *Int J Sports Med* 1992; S59-61.

19. Ono S, Westcott JY, Chang SW, et al. Endotoxing priming followed by high altitude causes pulmonary edema in rats. *J Appl Physiol* 1993; 74: 1534-1542.

20. Irwin DC, Rhodes J, Baker DC, et al. Atrial natriuretic peptide blockade exacerbates high altitude pulmonary edema in endotoxin primer rat. *High Altitude Medicine & Biology* 2001; 2: 349-360.

21. Engebretsen BJ, Irwin D, Valdez ME, et al. Acute hypobaric hypoxia (5486m) induces greater pulmonary HIF-1 activation in Hilltop compared to Madison rats. *High Altitude Medicine & Biology* 2007; 8: 312-321.

22. Kleinsasser A, Levin DL, Loeckinger A, et al. A pig model of High altitude pulmonary edema. *High Altituee Medicine & Biology* 2003; 4: 465-474.

23. Pottle D, and Gosselin LE. Impact of mechanical load on functional recovery after muscle reloading. *Med Sci Sports Exerc* 2000; 32: 2012-2017.

24. Sun JY, Guo WG, Ben Y, et al. Preventive effects of curcumon and dexamethasone on lung transplantation- associated lung injury in rats. *Crit Care Med* 2008; 36: 1205-1213.

25. Su X, Song YL, Jiang JJ, et al. The role of aquaporin-1 (AQP1) expression in a murine model of lipopolysaccharide-induced acute lung injury. *Respir Physiol Neurobiol* 2004; 142: 1-11.

26. Su X, Wang L, Song YL, et al. Inhibition of inflammatory response by ambroxol, a mucolytic agent, in a muring model of acute lung injury induced by lipopolysaccharide. *Intensive Care Med* 2004; 30: 133-140.

27. Riches PG, Sheldon J, Smith AM, et al. Overestimation of monoclonal immunoglobulin by immunochemical methods. *Ann Clin Biochem* 1991; 28: 253-259.

28. She J, Sun Q, Fan L, et al. Association of HLA genes with diffuse panbronchiolitis in Chinese Patients. *Respir Physiol Neurobiol* 2007; 157: 266-273.

29. Michel RP, Hakim TS, Smith TJ, et al. Quantitative morphology of permeability lung edema in dogs induced by alpha-naphthylthiourea. *Lab Invest* 1983; 49:412-419.

30. Briot R, Bayat S, Anglade D, et al. Monitoring the capillary-alveolar leakage in an A.R.D.S. model using broncho-alveolar lavage. *Microcirculation* 2008; 15: 237-249.

31. Ganter MT, Rous J, Miyazawa B, et al. Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. *Circ Res* 2008; 102:804-812.

32. Pittet JF, Griffiths MJ, Geiser T, et al. TGF-beta is a critical mediator of acute lung injury. *J Clin Invest* 2001; 107: 1537-1544.

33. Dehnert C, Berger MM, Mairbaurl H, Bartsch P. High altitude pulmonary edema: a pressure-induced leak. *Respir Physiol Neurobiol* 2007; 158: 266-273.

TABLES

Table 1

	NR	NE	HR	HE
PaO_2 (mmHg)	91.6±0.9	91.7±1.1	58.2±1.3**	55±1.2**
$PaCO_2$ (mmHg)	35.9±1.3	31.7±1.1	26.8±1.9*	24.6±1.3**
pH	7.39±0.01	7.39±0.01	7.46±0.02*	7.38±0.02
PA-aO_2	22.16±1.36	26.36±1.57	27.56±3.05	32.92±1.81*
Wet/Dry ratio	3.3±0.27	3.6±0.29	4.6±0.29*	5.8±0.32**, #
Total BAL protein (µg/ml)	302.8±24.4	318.3±22.6	488.4±28.5**	603.4±39.8**, #
BAL albumin (µg/ml)	188.3±25.1	200±28.6	321.9±26.2*	376.4±25.9**

BAL RBC (*10^5/ml)	13.9±1.4	19.5±3.1	45.9±6.5*	66.6±4.4**, #
BAL WBC (*10^5/ml)	4.1±0.6	4.4±0.7	7±0.5*	7.1±0.7*
BAL IgM (mg/dl)	0.09±0.05	0.08±0.05	0.08±0.06	0.06±0.05
BAL LTB4 (pg/ml)	0.07±0.02	0.07±0.02	0.07±0.02	0.07±0.006

Values are given as mean ± SEM

Table 2

	$PA\text{-}aO_2$	**BAL LTB4 (pg/ml)**	**Breaks (SEM)**	**neutrophile infiltration score**
Edema score	0.78*	0.21 (NS)	0.9*	0.34 (NS)
Breaks (SEM)	0.79*	0.11 (NS)		0.38 (NS)
Wet/Dry ratio	0.81*	0.24 (NS)	0.9*	0.35 (NS)
BAL albumin (µg/ml)	0.80*	0.29 (NS)	0.89*	
BAL RBC (*10^5/ml)	0.78*	0.16 (NS)	0.92*	
BAL LTB4 (pg/ml)	0.2 (NS)		0.11 (NS)	

***p<0.01**

NS: Non Significant (p>0.05)

TABLE LEGENDS

Table 1. Summary of arterial blood gas analysis, lung wet-dry weight ratio data and BAL analysis. ***Arterial blood gas analysis.*** PaO_2, ** $p < 0.01$ for the HE and HR groups *vs.* NE and NR groups. $PaCO_2$, ** $p < 0.01$ for the HE group *vs.* NR group; * $p < 0.05$ for the HR group *vs.* NR group. pH, * $p < 0.05$ for the HR group vs. HE, NE and

NR groups. PA-aO2, * $p < 0.05$ for the HE group vs. NR, NE and HR groups. ***Lung wet-to-dry weight ratio.*** ** $p < 0.01$ for the HE *vs.* NE and NR groups; * $p < 0.05$ for the HR group vs. NR group. ***Bronchoalveolar lavage.*** Total protein, ** $p < 0.01$ for the HE and HR group vs. NE and NR group, respectively. Albumin, ** $p < 0.01$ for the HE group vs. NE and NR group. * $p < 0.05$ for the HR group vs. NE and NR group. RBC, ** $p < 0.01$ for the HE group vs. NE and NR group; * $p < 0.05$ for the HR group vs. NE and NR group. # $p < 0.05$ for the HE group vs. HR group. WBC, * $p < 0.05$ for the HE and HR groups vs. NE and NR groups. BAL IgM, no significant differences among groups. BAL LTB4, no significant differences among groups.

Table 2. Correlation table among BAL measurements and histological parameters. R is provided for each couple of variables. The p values have been calculated using the Pearson test and are provided for each couple of variables. Variables abbreviations: RBC, red blood cell; LTB4, leukotriene B4; Beaks, averaged number of breaks observed per animal in electron microscopy; PA-aO_2, alveolar-arterial difference of oxygen partial pressure.

FIGURE LEGENDS

Figure 1. Lung histology using hematoxylin/eosin staining.

(A) NR rats. (B) NE rats. Slightly thickened alveolar septum and few red blood cells in the interstitium. (C) HR rats. The arrow indicates interstitial lung edema. (D-1) Non

survivors from the HE group. The arrow indicates alveolar edema with red blood cells. The slice presents a diffuse interstitial edema. (D-2) Survivors from the HE group. The arrow indicates alveolar edema with red blood cells. The slice presents a diffuse interstitial edema. Two magnifications (X200 and X400) of the same slice are displayed for each group.

FIGURE 1

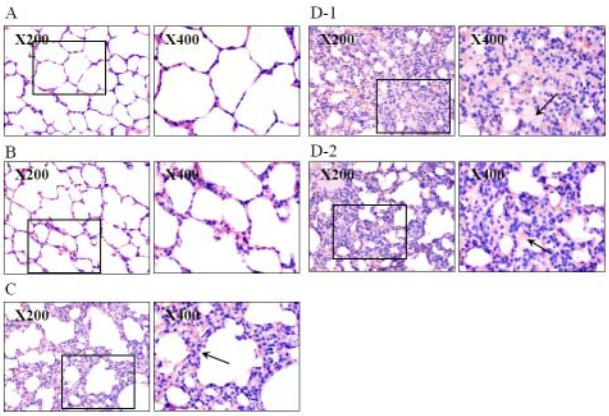

Figure 2. Lung injury scores from histology.

(A) Alveolar edema score, ** $p < 0.01$ for the HE group vs. HR, NE and NR groups. (B) Interstitial edema score, ** $p < 0.01$ for the HE and HR groups vs. NE and NR groups. (C) Hemorrhage score, ** $p < 0.01$ for the HE group vs. NR group. # $p < 0.05$ for the HE vs. NE group. (D) Neutrophil and macrophage infiltration score, * $p < 0.05$ for the HE group vs. NR group. N = 8 in each group. Data shown was mean ± SEM.

FIGURE 2

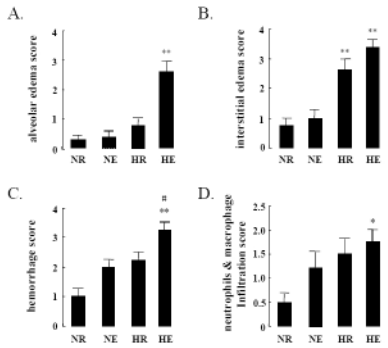

Figure 3. Electron micrograph.

(A) Control group (NR). Continuity of the blood-gas barrier and RBC in the capillary (scale bar = 1μm). (B) NE group. Continuity of the blood-gas barrier and RBC in the capillary (scale bar = 1μm). Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall. (C) HR group. Swelling of endothelial layer and epithelial lining (see arrows, scale bar = 2μm) and RBC in the capillary and alveoli. Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall. (D-1) HE group. Complete rupture of the blood-gas barrier including epithelium, endothelium and basement membrane (see arrows, scale bar = 1μm). Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall. (D-2) HE group. Disruption of endothelial layer (see arrows, scale bar = 2μm). Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall. (D-3) HE group. Swelling of epithelial lining (arrow, scale bar = 2μm) and RBC in the alveolar space. Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall (D-4) HE group. Disruption of epithelial lining (see arrows, scale bar = 1μm). Lanthanum nitrate was used as a tracer to highlight of alveolocapillary wall. **(E)** Summary of electron microscopy findings. The results are averaged numbers (±SEM**)** of alveolo-capillary disruption observed per surface unit of

slice. 3 slices were analyzed for each rat. ** $p < 0.01$ for the HE vs. HR, NE and NR groups.

FIGURE 3