Hypobaric hypoxia preconditioning attenuates acute lung injury during high-altitude exposure in rats via up-regulating heat-shock protein 70

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ABSTRACT
HHP (hypobaric hypoxia preconditioning) induces the overexpression of HSP70 (heat-shock protein 70), as well as tolerance to cerebral ischaemia. In the present study, we hypothesized that HHP would protect against HAE (high-altitude exposure)-induced acute lung injury and oedema via promoting the expression of HSP70 in lungs prior to the onset of HAE. At 2 weeks after the start of HHP, animals were exposed to a simulated HAE of 6000 m in a hypobaric chamber for 24 h. Immediately after being returned to ambient pressure, the non-HHP animals had higher scores of alveolar oedema, neutrophil infiltration and haemorrhage, acute pleurisy (e.g. increased exudate volume, increased numbers of polymorphonuclear cells and increased lung myeloperoxidase activity), increased pro-inflammatory cytokines [e.g. TNF-α (tumour necrosis factor-α), IL (interleukin)-1β and IL-6], and increased cellular ischaemia (i.e. glutamate and lactate/pyruvate ratio) and oxidative damage [glycerol, NOx (combined nitrate + nitrite) and 2,3-dihydroxybenzoic acid] markers in the BALF (bronchoalveolar fluid). HHP, in addition to inducing overexpression of HSP70 in the lungs, significantly attenuated HAE-induced pulmonary oedema, inflammation, and ischaemic and oxidative damage in the lungs. The beneficial effects of HHP in preventing the occurrence of HAE-induced pulmonary oedema, inflammation, and ischaemic and oxidative damage was reduced significantly by pretreatment with a neutralizing anti-HSP70 antibody. In conclusion, HHP may attenuate the occurrence of pulmonary oedema, inflammation, and ischaemic and oxidative damage caused by HAE in part via up-regulating HSP70 in the lungs.

INTRODUCTION
In hypoxic conditions, such as at high altitude, hypoxia-induced pulmonary hypertension facilitates alveolar fluid accumulation, and the hypoxia-induced impairment of the respiratory transepithelial sodium transport interferes with alveolar fluid clearance [1]. These alterations facilitate pulmonary oedema. Human studies suggest that

Key words: acute lung injury, heat-shock protein 70 (HSP70), high altitude, hypobaric hypoxia, preconditioning.
Abbreviations: ATA, atmosphere absolute; Ab, antibody; BALF, bronchoalveolar fluid; DHBA, dihydroxybenzoic acid; HAE, high-altitude exposure; HAPE, high-altitude pulmonary oedema; HHP, hypobaric hypoxia preconditioning; HSP70, heat-shock protein 70; IL, interleukin; LPS, lipopolysaccharide; MPO, myeloperoxidase; NBA, normobaric air; NOx, combined nitrate + nitrite; PMN, polymorphonuclear leucocyte; ROS, reactive oxygen species; TBS-T, Tris-buffered saline containing 0.1 % Tween 20; TNF-α, tumour necrosis factor-α.
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increased sympathetic activation of α-adrenergic efferent pathways [2] are associated with endothelin [3], ANP (atrial natriuretic peptide) [4] and reduced bioavailability of the endogenous vasodilator NO [5–9]. In addition, the mitochondrial formation of ROS (reactive oxygen species) is recognized as the primary stimulus underlying pulmonary hypertension [10,11].

Chronic hypoxic preconditioning is known to induce local and systemic adaptive responses such as an increase in blood haemoglobin concentrations and tissue oxygen delivery [12,13]. Chronic hypoxic preconditioning also improves brain tissue oxygenation by increasing brain capillary density [14,15]. Furthermore, mild hypoxic preconditioning increases the neuronal resistance to subsequent severe hypoxia [16,17]. In addition, brief hypoxia causes the overexpression of HSP (heat-shock protein)-70 as well as tolerance to focal cerebral hypoxia in rats [18]. Our previous results have shown that the observed benefit of HHP (hypobaric hypoxia preconditioning) was related to an increase in HSP70 overexpression in both the kidneys and lungs during heatstroke [19]. However, whether HHP protects against pulmonary vasoconstriction and systemic accumulation of oxidative/nitrosative/inflammatory stress biomarkers at high altitude via promoting the appearance of HSP70 in lungs prior to the onset of HAE (high-altitude exposure) remains unknown [20].

Therefore our primary aim of the present study was to examine the temporal effects of hypoxia on alveolar oedema, neutrophil infiltration and haemorrhage scores, acute pleurisy and levels of pro-inflammatory cytokines, cellular ischaemia and oxidative damage markers in the lungs during an ascent to HAE in rats without or with HHP. The secondary aim was to determine whether the beneficial effect of HHP in reducing acute lung injury and oedema during HAE is due to the induction of HSP70 in the lungs prior to the onset of HAE.

**MATERIALS AND METHODS**

**Animals**

Adult Sprague–Dawley rats (weight 254 ± 12 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China. The animals were housed four in a group at an ambient temperature of 22 ± 1°C, with a 12-h light/dark cycle. Rat chow pellets and tap water were available ad libitum. All protocols were approved by the Animals Ethics Committee of the Chi Mei Medical Center in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. At the end of the experiments, control rats and any rats that had survived HAE were killed with an overdose of urethane.

**HHP**

Rats were randomly assigned to one of the following three groups: (i) HHP group [18.3 % O2 at 0.66 ATA (atmosphere absolute) for 5 h/day on 5 consecutive days for 2 weeks], (ii) non-HHP group (21 % O2 at 1.0 ATA for 5 h/day for 5 consecutive days for 2 weeks); and (iii) NBA (normobaric air) group (21 % O2 at 1.0 ATA). At 2 weeks after the start of HHP, the HHP rats were subjected to simulated HAE (see below).

**Inhibition of HSP70 activity**

The neuroprotective role of HSP70 in the rat brain has been studied previously by using an anti-HSP70 Ab (antibody) [20,21]. A neutralizing polyclonal rabbit anti-(mouse HSP70) Ab (0.2 mg/kg of body weight; SPA-812E; Assay Designs) dissolved in non-pyrogenic sterile saline was injected intravenously 24 h prior to simulated HAE.

**Simulated HAE model**

HHP rats or non-HHP rats were randomly exposed to a simulated HAE (9.8 % O2 at 0.47 ATA) of 6000 m (or 19685 ft) in a hypobaric chamber (Institute of Aviation Medicine) for 24 h [18]. The temperature of the hypobaric chamber was maintained at 25 ± 1°C and humidity at 40–50 %, with an air flow rate of 4 litres/h and a barometric pressure of 355 mmHg. The animals were provided with adequate quantities of food and water during hypoxic exposure.

**Experimental protocol**

In experiment 1, determination of the protein expression of HSP70 in lung tissues was performed in non-HHP rats (n = 8), HHP rats (n = 8) and HHP + HSP70 Ab rats (n = 8) 24 h before the start of HAE, as shown in Figure 1.

In experiment 2, HAE was randomly performed in non-HHP rats (n = 8), HHP rats (n = 8) and HHP + HSP70 Ab rats (n = 8), and the effects on acute lung injury scores were assessed 24 h after HAE. Non-HAE non-HHP rats were used as controls.

In experiment 3, HAE was randomly performed in non-HHP rats (n = 8), HHP rats (n = 8) and HHP + HSP70 Ab rats (n = 8), and the effects on exudate volume, the number of PMNs (polymorphonuclear leucocytes) and MPO (myeloperoxidase) activity in lung tissues were assessed 24 h after HAE. NBA rats (no HAE or HHP) were used as controls.

In experiment 4, HAE was randomly performed in non-HHP rats (n = 8), HHP rats (n = 8) and HHP + HSP70 Ab rats (n = 8), and the effects on the BALF (bronchoalveolar fluid) levels of TNF-α (tumour necrosis factor-α), IL ( interleukin)-6 and IL-1β were assessed 24 h after HAE. NBA rats (no HAE or HHP) were used as controls.
In experiment 5, HAE was randomly performed in non-HHP rats \( (n = 8) \), HHP rats \( (n = 8) \) and HHP + HSP70 Ab rats \( (n = 8) \), and the effects on the BALF levels of glutamate, glycerol, 2,3-DHBA (dihydroxybenzoic acid) and NO\( _x \) (combined nitrate + nitrite) were assessed 24 h after HAE. NBA rats (no HAE or HHP) were used as controls.

**Protein preparation**

Peripheral lung tissues were frozen in liquid nitrogen and then stored at \(-80^\circ C\) until analysed. The tissue was homogenized in ice-cold isolation solution (250 mmol/l sucrose, 10 mmol/l triethanolamine, 1 \( \mu \)g/ml leupeptin and 0.1 mg/ml PMSF). Homogenates were centrifuged at 12000 \( g \) for 10 min at \( 4^\circ C \) to separate incompletely homogenized tissue. The supernatants were obtained and the protein concentrations were measured using a protein assay kit (Sunbio). For deglycosylation of proteins, an N-glycosidase F deglycosylation kit (Roche) was used.

**Western blot analysis**

Total proteins (50 \( \mu g \)/sample) were diluted in 5 × loading buffer [0.25 mol/l Tris/HCl (pH 6.8), 10% SDS, 0.5% Bromophenol Blue, 50% glycerol and 0.5 mol/l dithiothreitol] and then boiled for 5 min. SDS/PAGE was carried out on 12% gradient gels. The proteins were electrophoretically transferred on to PVDF membranes treated previously with methanol and blocked for 1 h at room temperature (24°C) in TBS-T (Tris-buffered saline containing 0.1% Tween 20) containing 5% non-fat dried milk. Membranes were incubated overnight at 4°C with an anti-HSP70 antibody (1:40000 dilution; Santa Cruz Biotechnology) in TBS-T containing 5% non-fat dried milk. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-labelled anti-rabbit antibody (Santa Cruz, 1:3000) 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 an anti-\( \beta \)-actin antibody (1:1000 dilution; Santa Cruz Biotechnology), which was visualized via enhanced chemiluminescence as described above. For quantification, Western blots were scanned using a Minolta scanner and Adobe Photoshop software. The labelling density was quantified using Lab-Works software (UVP). The relative density of the HSP70 band was normalized to the density of the \( \beta \)-actin band to represent the amount of HSP70 protein. The ratio of non-HHP group was regarded as 100%, and the results for the HHP and HAE groups are expressed as a percentage of the value from the non-HHP group.

**Lung morphology**

At the end of the experiments, the animals were killed before their lungs were excised en bloc. Lung tissues were fixed in 10% buffered formalin for 24 h and then embedded in paraffin and cut into 3-\( \mu m \)-thick sections. Sections were stained with haematoxylin and eosin, and images were taken with an Olympus BX51 microscope using a \( \times 40 \) objective. A lung injury scoring method was applied to quantify the changes in lung architecture visible by light microscope. The degree of microscopic injury was scored based on the following variables: alveolar and interstitial oedema, neutrophil infiltration and haemorrhage. The severity of injury was graded for each variable: 0, no injury; 1, injury to 25% of the field; 2, injury to 55% of the field; 3, injury to 75% of the field; and 4, diffuse injury [22]. All samples were analysed based on a scaled grading system by a pathologist who was blinded to the experimental protocol and the region of sampling. A total of three slides from each lung sample were randomly screened, and the mean was taken as the representative values of the sample. For presentation, representation examples were chosen which
were observed in all of the preparations from the same treatment.

**Determination of exudate volume and PMNs**

The chest was carefully opened, and the pleural cavity was washed with 2 ml of saline solution containing heparin (5 units/ml) and indomethacin (10μg/ml). The exudates and the washing solution were removed by aspiration and the total volume was measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume recorded. Cells were counted with the aid of a haemocytometer, and PMN populations were found to contain at least 95% PMNs as demonstrated by cytoospin and differential stain analysis (vital Trypan Blue stain).

**Determination of pro-inflammatory cytokines, glutamate, glycerol, 2,3-DHBA and NOx**

The lungs were lavaged by installation of 5 ml of saline at room temperature through a polyethylene tube (2.0 nm in diameter) into the trachea. The saline installed into the lungs was then withdrawn. After centrifugation (830 g for 10 min), the BALF supernatant was frozen at −80°C for cytokine assay. In separate experiments, the BALF supernatant was collected for glutamate, glycerol, 2,3-DHBA and NOx determination. The concentrations of TNF-α, IL-1β and IL-6 in the BALF were determined using double-antibody sandwich ELISAs (R&D Systems), according to the manufacturer’s instructions. Absorbances were read on a plate reader set at 450 nm for TNF-α, IL-1β and IL-6. The concentrations of cytokines in the samples were calculated from the standard curve multiplied by the dilution factor and are expressed in pg/ml. For determination of glutamate and glycerol, samples were injected into a CMA600 microdialysis analyser (Carnegie Medicine) [23]. It should be stressed that there was no repeated BALF used for determination of markers of oxidative/nitrostative stress.

NO is an unstable molecule that is easily degraded into nitrite and nitrate ions [24]. These stable NO metabolites have been reported to reflect the levels of regional NO production/release [24]. Nitrite and nitrate levels were measured using an HPLC-NO detector system (ENO-10; Eicom), as reported previously [25]. In brief, nitrite and nitrate were separated on a reverse-phase column (NO-PAK, 4.6 mm × 50 mm; Eicom), and nitrate was reduced to nitrite by passage through a reduction column (NO-RED; Eicom). Nitrite was determined as the azo dye compound formed by the Griess reaction using a spectrophotometer. These oxidative NO products were also evaluated as NOx.

The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxylation of sodium salicylates by hydroxyl radicals, leading to the production of 2,3-DHBA and 2,5-DHBA [26,27]. A Ringer’s solution containing 0.5 mmol/l sodium salicylates was perfused through the microdialysis probe at a constant flow rate (1.2 μl/min). An Alltima reverse-phase C18 column (150 mm × 1 mm internal diameter, particle size 5 μm; BAS) was used to separate the DHBAs, and the mobile phase consisted of a mixture of 0.1 mol/l chloroaetic acid, 26.87 nmol/l disodium EDTA, 688.16 nmol/l sodium octylsulfate and 10% acetonitrile (pH 3.0). The retention times of 2,3-DHBA and 2,5-DHBA were 8.1 and 6.0 min respectively.

**Statistical analysis**

All quantitative data were evaluated for Gaussian (normal) distribution. Values are the means ± S.D. and were analysed with one-way ANOVA, followed by the Neumann–Keuls post-hoc test if P < 0.05. Relationships between lung injury and the expression of HSP70 were analysed by calculating a Pearson product-moment correlation coefficient. A value of P < 0.05 was considered statistically significant. All data were analysed with SigmaPlot for Windows version 11.0 (Systat Software).

**RESULTS**

**HHP increased HSP70 protein in lungs**

Western blot analysis revealed that expression of HSP70 protein in lung tissues from the HHP group was significantly higher than those from the non-HHP group (P < 0.01; Figure 2). On the other hand, the expression of HSP70 protein in lung tissues from the HHP + NS group were significantly lower than those from the HHP group (P < 0.01; Figure 2).

**HHP attenuated HAE-induced acute lung injury**

The lungs of rats in the non-HHP group showed congestion, swelling and haemorrhage at the opening of the chest when evaluated 24 h after the start of HAE. Histological examination revealed marked interstitial oedema (indicated by thickened interalveolar septum, capillary expansion, hyperaemia and pink exudation in alveolar space) in all of the sections from this group. All of the injury scores, including oedema, neutrophil infiltration and haemorrhage, were significantly increased in the non-HHP group compared with those from the NBA controls (Figure 3). However, the animals in the HHP group had significantly lower scores of acute lung injury.
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Figure 2. Western blot analysis of HSP70 in the lungs of non-HHP, HHP and HHP + HSP70 Ab rats
(A) Representative immunoblot of HSP70. (B) Quantification of HSP70 expression. Fold-change values represent the mean of eight samples (n = 8) divided by the mean of the eight controls (n = 8). Results are presented as means ± S.D. *P < 0.01 compared with the non-HHP group; +P < 0.01 compared with the HHP group.

when compared with those of the non-HHP group (Figure 3).

**HHP reduced acute pleurisy and the increase in lung MPO activity after HAE**

The exudate volume, numbers of PMNs in the BALF and lung MPO activity in the non-HHP group were significantly higher 24 h after HAE than for the NBA controls (Figure 4). However, both acute pleurisy and the increase in MPO activity after HAE were significantly reduced by HHP (Figure 4).

**HHP reduced the overproduction of pro-inflammatory cytokines after HAE**

The BALF levels of TNF-α, IL-6 and IL-1β after HAE for non-HHP rats were significantly higher than those of NBA controls (Figure 5). However, HHP significantly reduced the HAE-induced overproduction of these pro-inflammatory cytokines in the BALF.

**HHP reduced the BALF levels of glutamate, glycerol, 2,3-DHBA and NOx after HAE**

The BALF levels of glutamate, glycerol, 2,3-DHBA and NOx in non-HHP rats were significantly higher 24 h after the start of HAE than they were for NBA controls (Figure 6). However, the HAE-induced increase in BALF levels of all of these parameters were significantly reduced by HHP before the start of HAE (P < 0.05).

**HHP-induced beneficial effects in reducing acute lung injury, inflammation and ischaemic and oxidative damage during HAE are inhibited by an anti-HSP70 Ab**

The beneficial effects of HHP in reducing HAE-induced acute lung injury, overproduction of pro-inflammatory cytokines in the BALF, pleurisy, increased lung MPO activity, and ischaemia and oxidative damage in the lungs was significantly ameliorated by treatment with the neutralizing anti-HSP70 Ab (Figures 3–6).

**Inverse correlation between acute lung injury score and lung HSP70 expression**

Pearson correlation analysis revealed that there was an inverse correlation between acute lung injury scores (oedema, neutrophil and haemorrhage scores) and HSP70.
DISCUSSION

When the non-HHP rats were exposed to a simulated HAE of 6000 m in a hypobaric chamber for 24 h, they displayed higher scores of alveolar oedema, neutrophil infiltration and haemorrhage compared with those of the NBA controls. Additionally, acute pleurisy (indicated by increased exudate volume and an increase in numbers of PMNs), increased BALF levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), cellular ischaemia markers (glutamate and lactate/pyruvate ratio), damage markers (glycerol) and oxidative damage markers (NOx and 2,3-DHBA), and increased MPO activities in the lungs were observed in non-HHP rats after HAE. Furthermore, a Pearson correlation analysis revealed that there was an inverse correlation between lung interstitial oedema scores and HSP70 protein expression in the lungs of these rats 24 h after HAE. HHP, in addition to increasing HSP70 expression in the lung tissue, significantly reduced HAE-induced acute lung injury in rats. Furthermore, the beneficial effects of HHP in improving lung injury during HAE were reversed by a neutralizing anti-HSP70 Ab. Thus it appears that HHP may attenuate HAE-induced acute lung injury or oedema via up-regulating lung HSP70. However, it remains unclear to what extent HHP influenced physical activity levels and indeed food intake, which may be considered potential confounding factors.

In rats, LPS (lipopolysaccharide) administered intraperitoneally and intratracheally, as well as intranasally, evoked an acute lung injury in rats [28,29]. Intraperitoneal administration of LPS also caused an increase in TNF-α concentrations in BALF, MPO activities and the wet/dry ratio in lung tissues when compared with saline-treated controls [30]. TNF-α is believed to be an important mediator in the early cascade of LPS-induced lung inflammation [31]. In patients with ARDS (adult respiratory distress syndrome), the BALF levels of IL-1β were also elevated [32]. These observations suggest that the occurrence of acute lung oedema is associated with lung inflammatory responses. As shown in the present study, the full spectrum of lung inflammatory syndromes (including overproduction of

**Figure 4** Exudate volume, number of PMNs and MPO activity in lung tissues from NBA, non-HHP, HHP and HHP + HSP70 Ab rats
Open bar, NBA rats; stripped bar, non-HHP rats; hatched bar, HHP rats; and closed bar, HHP + HSP70 Ab rats. Results are means ± S.D. (n = 8). * P < 0.05 compared with the NBA group; † P < 0.05 compared with the non-HHP group; § P < 0.05 compared with the HHP + HSP70 Ab group.

**Figure 5** TNF-α, IL-1β and IL-6 levels in BALF from the NBA, non-HHP, HHP and HHP + HSP70 Ab rats
Open bar, NBA rats; stripped bar, non-HHP rats; hatched bar, HHP rats; and closed bar, HHP + HSP70 Ab rats. Results are means ± S.D. (n = 8). * P < 0.05 compared with the NBA group; † P < 0.05 compared with the non-HHP group; § P < 0.05 compared with the HHP + HSP70 Ab group.
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pro-inflammatory cytokines, acute pleurisy and PNM accumulation) caused by HAE could be reduced by HHP. Pretreating with the neutralizing anti-HSP70 Ab significantly attenuated the beneficial effects of HHP in reducing lung inflammation and oedema. Nevertheless, prospective human studies measuring cytokines in plasma and other related parameters found no evidence for inflammation before or with the onset of HAPE (high-altitude pulmonary oedema) [33,34]. Swenson et al. [7] showed further that early HAPE was characterized by pulmonary hypertension that led to pulmonary oedema, with normal levels of leucocytes, cytokines and eicosanoids. These results demonstrated that inflammation may be not necessary or causal for the leak in early HAPE in humans.

The reduction in barometric pressure and the consequent fall in the $P_{O_2}$ (partial pressure of $O_2$) at higher altitudes leads to hypobaric hypoxia. If the hypoxia is severe or sustained, as in the present study, a fall in the $O_2$ saturation in multiple organs is inevitable, leading to multiorgan dysfunction or failure (including the lungs). Indeed, it has been reported that hypoxia stimulated glutamate release, which caused excitotoxicity of cells by increasing intracellular Ca$^{2+}$ levels, activating NOS (NO synthase) and generating NO$_x$ [33]. The hypoxia-induced generation of RNS (reactive nitrogen species) and ROS could cause protein oxidation [34], lipid oxidation [35] or cell death. In a previous report [36], an increase in oxidative stress in the hippocampus, as detected by an increase in free radical production and NO levels, was observed in rat exposed to a simulated high altitude equivalent to 6100 m in an animal decompression chamber for 3–7 days. Our present results also showed that the non-HHP rats had an increase in free radical production and NO$_x$ level in the BALF after a simulated HAE of 6000 m (9.8 % $O_2$ at 0.47 ATA) for 24 h. 2,3-DHBA and NO$_x$ are two well-known markers of cellular oxidative damage markers [37,38]. Again, the HAE-induced oxidative damage in lungs was significantly reduced by HHP in rats. Additionally, the beneficial effects of HHP in reducing oxidative damage in the lung could be significantly attenuated by pretreating with a neutralizing anti-HSP70 Ab. However, the question whether it is the hypoxia or hypobaria that is driving the protective benefits of HHP warrants future studies. Again, we have to give the linear relationship (dose–response) in future studies to show that the fundamental mechanism associated with the pulmonary-protective benefits of HHP could simply be related to the fact that animals were less hypoxic when exposed to HAE.

It is well documented that glutamate and the lactate/pyruvate ratio are two markers of cellular ischaemia, whereas glycerol is a marker of how severely cells are affected by the ongoing pathology [23]. Our previous studies also demonstrated that animals with traumatic brain injury had higher values of extracellular levels of glutamate, lactate/pyruvate ratio and glycerol in the ischaemic cortex [39,40]. Our present findings have shown that increased levels of glutamate, lactate/pyruvate ratio and glycerol in the BALF of non-HHP rats after a simulated HAE were observed. The HAE-induced increase in cellular ischaemia and damage markers was associated with an increase in lung injury scores, which was reduced significantly by HHP. Again, pretreatment with the neutralizing anti-HSP70 Ab significantly

Table 1  Pearson correlation analysis between pulmonary scores of oedema, haemorrhage and neutrophil infiltration and pulmonary HSP70 expression in rats ($n=8$) exposed to HAE for 24 h

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$R$</th>
<th>$P$</th>
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<tbody>
<tr>
<td>Oedema</td>
<td>0.682</td>
<td>0.000</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>0.624</td>
<td>0.000</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>-0.725</td>
<td>0.000</td>
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Figure 6  Glutamate, glycerol, 2,3-DHBA and NO$_x$ levels in BALF from the NBA, non-HHP, HHP and HHP + HSP70 Ab rats

Open bar, NBA rats; stripped bar, non-HHP rats; hatched bar, HHP rats; and closed bar, HHP + HSP70 Ab rats. Results are means ± S.D. ($n=8$). * $P<0.05$ compared with the NBA group; † $P<0.05$ compared with the non-HHP group; ‡ $P<0.05$ compared with the HHP + HSP70 Ab group.

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abolished the HHP-induced beneficial effects in reducing lung ischaemia and damage.

Evidence has suggested that aquaporin 1 and aquaporin 5 play roles in the formation of HAPE [41,42]. Pearson correlation analysis revealed that there was an inverse correlation between lung injury scores and mRNA and protein expression of aquaporin 1 and aquaporin 5 in the lungs of rats [18]. During HAE, the lower expression of both aquaporin 1 and aquaporin 5 might cause the formation of pulmonary oedema. Again, it is not known whether HHP-induced overexpression of lung HSP70 improves HAPE by modulating the mRNA and protein expression of aquaporin 1 and aquaporin 5 in the lungs.

AUTHOR CONTRIBUTION
Hung-Jung Lin, Mao-Tsun Lin and Ching-Ping Chang participated in the design of the study and drafted the manuscript. Hung-Jung Lin and Ching-Ping Chang made substantial contributions to the conception and design of the study, acquisition of the data, and analysis and interpretation of the results. Ko-Chi Niu, Chia-Ti Wang and Zhuo Li made substantial contributions to the conception and design of the study. Mao-Tsun Lin and Chunjin Gao oversaw the study and gave final approval of the version to be published.

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