

# Penethylidine hydrochloride exerts protective effects in rats with acute lung injury via the Fas/FasL signaling pathway

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**Abstract.** Acute lung injury (ALI) is a critical syndrome that is associated with high morbidity and mortality rates. The activation of the Fas/Fas ligand (FasL) signaling pathway may be an important pathophysiological mechanism during ALI development. Penethylidine hydrochloride (PHC) has been revealed to exhibit anti-apoptotic properties and may attenuate the observed systemic inflammatory response. The present study was performed to elucidate the molecular mechanism of PHC in the regulation of the Fas/FasL signaling pathway in rats with ALI. An ALI rat model was constructed by inducing blunt chest trauma and hemorrhagic shock (T/HS), with PHC administration prior to or following T/HS. At 6 h following T/HS, blood samples and lung tissues were collected. Western blotting, arterial blood gas analysis, ELISA, hematoxylin and eosin staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining and biochemical indicator analysis were performed to determine the degree of lung injury and the key signaling pathways associated with lung damage. The results indicated that the administration of PHC following T/HS effectively attenuates lung injury by improving pulmonary oxygenation, decreasing histopathological damage, decreasing polymorphonuclear neutrophil count and decreasing Fas, FasL, caspase-8, caspase-3, tumor necrosis factor- $\alpha$ , interleukin (IL)-6 and IL-1 $\beta$  expression.

The results indicated that PHC exhibits anti-apoptotic functions and exerts protective effects in ALI rats induced by T/HS, which may be attributed to the inhibition of the Fas/FasL signaling pathway.

## Introduction

Blunt chest trauma and hemorrhagic shock (T/HS) occurs in patients with poly-trauma (possibly following vehicular accidents) and is a leading risk factor in the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (1,2). T/HS exhibits high morbidity and mortality rates (3,4). Furthermore, it is well known that apoptosis is involved in distinct types of ALI. Experimentally, Messer *et al* (5) and Thakkar *et al* (6) demonstrated that the activation of apoptosis via the Fas/Fas ligand (FasL) signaling pathway was important for the development of ALI caused by trauma or hemorrhagic shock. Clinically, Glavan *et al* (7) revealed that the content of soluble Fas and FasL was increased in pulmonary edema fluid, which was strongly associated with increased morbidity and mortality in patients with ALI. Additionally, Herrero *et al* (8) determined that the inhibition of the Fas/FasL signaling pathway alleviated ALI/ARDS damage.

Penethylidine hydrochloride (PHC) is a novel anti-cholinergic drug that was first developed by the Beijing Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (Beijing, China), which can be applied in anti-apoptosis and anti-inflammation treatment (9,10). Recently, an increasing number of studies have indicated that PHC may alleviate lung injuries by inhibiting ALI-induced apoptosis and inflammation (11-13). Furthermore, Wang *et al* (14) revealed that PHC mitigates lung injury by regulating the expression of Bcl-2-associated X (bax) and B-cell lymphoma-2 (Bcl-2) in a rat model of ALI following blunt chest trauma. Cui *et al* (15) also demonstrated that PHC pre-treatment reduced the expression of bax and caspase-3, decreased the indices of apoptosis and pulmonary vascular resistance, and improved PaO<sub>2</sub>/FiO<sub>2</sub> and Bcl-2/bax ratios in pigs with dichlorvos-induced ALI. However, the underlying mechanism of PHC in ALI requires further elucidation. The current study aimed to determine the effect of PHC on ALI as well as associated signaling pathways.

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**Abbreviations:** ALI, acute lung injury; PHC, penethylidine hydrochloride; ARDS, acute respiratory distress syndrome; T/HS, blunt chest trauma and hemorrhagic shock

**Key words:** penethylidine hydrochloride, Fas, Fas ligand, apoptosis, acute lung injury

## Materials and methods

**Animals.** A total of 40 Male Sprague-Dawley rats (age, 8-10 weeks) with a body weight of 245-275 g were obtained from the Hunan Institute for Biologic Sciences (Hunan, China; certificate no. SCXX 2009-0004) under specific pathogen-free conditions. The current study was approved by Medical Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China) and was performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were housed under a 12 h light/dark cycle at a temperature of 22°C and a humidity of 50-60% with free access to food and water.

**T/HS model.** Rats were anesthetized with intraperitoneal (IP) injections of sodium pentobarbital (30 mg/kg). To establish a rat model of blunt chest trauma, the current study generated an isolated bilateral lung contusion as described previously (16). Following anesthesia induction, a hollow cylinder (weight, 300 g) was dropped from a defined height (83.3 cm). The cylinder was encased in a vertical stainless steel tube, which was positioned on a platform. The precordial shield directed the impact force bilaterally to the lungs to prevent cardiac trauma (impact energy, 2.45 J). This experiment was performed by our laboratory according to a previous study by Raghavendran *et al* (17). A rat model of nonlethal hemorrhagic shock was then established based on a previous study (12). The femoral artery and vein were cannulated with polyethylene (PE-50) tubing, and blood flowed via tubing, which was attached to the monitor (IntelliVue MP40; Phillips Medical Systems B.V., Eindhoven, The Netherlands), until the average arterial blood pressure reached 35±5 mm Hg. This pressure was then maintained for 60 min. Following a hypotensive period of 60 min, rats were resuscitated via the transfusion of removed blood with Ringer's lactate solution [Baxter Healthcare (Tianjin) Company, Ltd., Tianjin, China; Composition, 6.0 g/l Na<sup>+</sup>, 6.0; 0.3 g/l K<sup>+</sup>; 0.2 g/l Ca<sup>2+</sup>, 2H<sub>2</sub>O and Cl] twice over a period of 60 min. ALI was defined as a PaO<sub>2</sub>/FiO<sub>2</sub> of <300 mm Hg.

**Experimental protocols.** A total of 40 rats were randomly divided into 4 equal groups (each, n=10): A sham group, a T/HS group, a PHC1 group and a PHC2 group. According to previous studies (12,16), rats of the PHC1 group were infused with 2 mg/kg PHC 30 min prior to blunt chest trauma. PHC2 group rats were infused with 2 mg/kg PHC 60 min following hemorrhagic shock. The Sham and T/HS groups received the same volume (0.5 ml) of 0.9% normal saline solution. Prior to blunt chest trauma, rats were anesthetized and the femoral artery and vein were cannulated with polyethylene (PE-50) tubing for continuous invasive pressure monitoring and to establish venous access. Rats in the sham group were subjected to the same experimental procedures, including the cannulation of femoral artery and vein, but without T/HS. All animals were sacrificed under deep anesthesia (50 mg/kg IP pentobarbital) and exsanguinated from the right carotid artery 6 h following T/HS. Blood and lung samples were then collected.

**Arterial blood gas analysis.** Following the induction of T/HS for 6 h, rats were anesthetized with an IP injection of

pentobarbital (50 mg/kg) and arterial blood was obtained from the right carotid artery (1 ml each). Samples were immediately analyzed using a blood gas analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) for the determination of PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub>.

**Polymorphonuclear neutrophils (PMNs) and protein in BALF.** Following rat sacrifice, the trachea was cannulated and lavaged. Bronchoalveolar lavage fluid (BALF) was prepared by washing the lungs three times with 2 ml PBS. BALF was centrifuged at 400 x g for 10 min at 4°C to pellet cells. BALF supernatant was then used for protein analysis. The cell pellet was resuspended in PBS and then the BALF cell counts were performed following trypan blue exclusion. An aliquot of pooled BALF (50 µl) from each rat was diluted 1:1 with trypan blue dye (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the total number of cells was counted using a standard hemocytometer. To analyze differential cell counts, 100 µl of BALF from each rat was centrifuged 1,000 x g for 10 min at 4°C using a Cytospin (Thermo Fisher Scientific, Inc.). After slides were dried, cells were fixed with 3% glutaraldehyde in PBS for 15 min at room temperature, and stained using Wright Stain solution (cat. no. 32857; Sigma-Aldrich, USA; incubated for 30 sec at room temperature) according to the manufacturer's instructions, and then differential cell counts were obtained by manually counting 200 cells per rat as previously described (18). BALF protein content was determined via a bicinchoninic acid (BCA) assay and absorbance was measured at 595 nm.

**Hematoxylin and eosin (H&E) staining and acute lung injury score.** Following animal sacrifice through bleeding from the right carotid artery at 6 h following T/HS, lung tissue samples were harvested immediately. Right middle-lung specimens were fixed using 10% formalin at room temperature for 24 h, sectioned (5 µm) and stained with H&E (hematoxylin staining for 10 min and eosin staining for 2 min; each at room temperature). Sections were observed under a light microscope with a magnification of x100. Sections were then evaluated and graded for the presence of interstitial neutrophilic infiltrate, intra-alveolar hemorrhage and pulmonary edema with a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

**Western blotting.** Western blot analysis was performed to determine Fas, FasL, caspase-3 and caspase-8 protein levels in rat lung tissues. Lung tissues were homogenized and lysed in lysis buffer [25 mM Tris-HCl (pH 7.6), 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Protein concentration was determined using a BCA protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) and equal quantities (50 µg) of protein were loaded per lane on a 14% SDS-PAGE gel. Samples were separated electrophoretically and then transferred to polyvinylidene difluoride membranes. Subsequently, membranes were blocked with 5% skimmed milk (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature for 2 h on a rotary shaker (60 rpm). Samples were then incubated with the following primary antibodies overnight at 4°C: Rabbit anti-Fas (1:1,000; cat. no. sc-21730; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-FasL

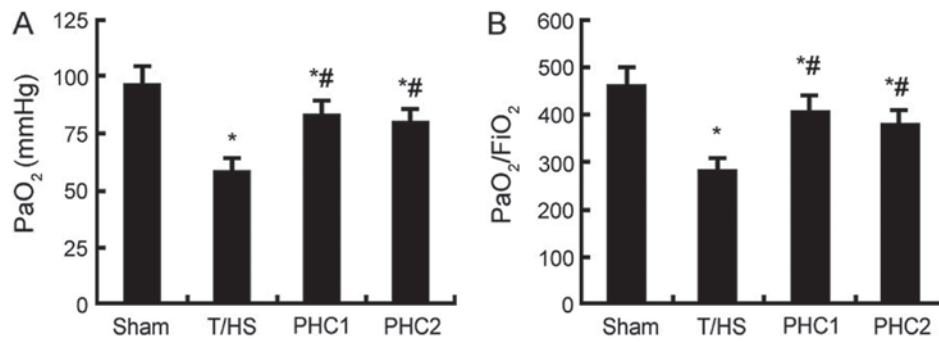


Figure 1. Effects of PHC on PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub>. (A) PaO<sub>2</sub> and (B) the PaO<sub>2</sub>/FiO<sub>2</sub> ratio in the T/HS, PHC1 and PHC2 group were significantly decreased compared with the sham group. PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> ratios were also increased in the PHC1 and PHC2 groups compared with the T/HS group. Data are expressed as the mean ± standard error of the mean. \*P<0.05 vs. the sham group; #P<0.05 vs. the T/HS group. PHC, penhexyclidine hydrochloride; T/HS, blunt chest trauma and hemorrhagic shock.

(1:1,000; cat. no. sc-834; Santa Cruz Biotechnology, Inc.), anti-caspase-3 (1:500; cat. no. 9662), anti-caspase-8 (1:500; cat. no. 9662; both Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin (1:1,000; cat. no. ab8227; Abcam, Cambridge, UK). Subsequently, membranes were washed with TBS-T three times and incubated with peroxidase-conjugated secondary antibodies (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Membranes were then washed three times with TBST solution and the blotted protein bands were observed using enhanced chemiluminescence (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK) and exposed to Kodak X-ray film (Kodak Biomax; cat. no. Z350400, Sigma-Aldrich; Merck KGaA). Proteins bands were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Immunohistochemistry.** The expression of Fas and FasL were determined via immunohistochemistry. Lung tissues were fixed in 4% paraformaldehyde for 24 h at 4°C, dehydrated, embedded in paraffin and subsequently cut into 5 mm slices. Sections were incubated with rabbit polyclonal anti-Fas (1:100; cat. no. sc-21730) and FasL (1:100; cat. no. sc-834; both Santa Cruz Biotechnology, Inc.) antibodies at 4°C for 15 h. Samples were then incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (1:250; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature and diluted in blocking solution (5% bovine serum albumin; cat. no. B2064; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Biotin-peroxidase and diaminobenzidine were used as substrates for the color reaction. The mean optical density of Fas and FasL positive cells from each section were analyzed using image cytometry with HIPAS-2000 image analysis software (Wuhan Qianli Technical Imaging Co. Ltd., Wuhan, China). The number of positive microvessels in each section was counted in 10 microscopic fields (at magnification, x400) under a light microscope (BX51; Olympus Corporation, Tokyo, Japan). The specificity of immunohistochemical staining was tested using PBS at the same dilution. Tissue sections in the sham group were used as negative controls.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay.** Apoptotic cells were

stained via the TUNEL technique using an apoptosis detection kit (cat. no. APOAF-50TST; Sigma-Aldrich; Merck KGaA) following the manufacturer's protocol. The sections were added onto a coverslip with DPX mounting medium (cat. no. 06522; Sigma-Aldrich; Merck KGaA). The total number of cells and the number of positive cells were counted in two sections from each animal (at magnification, x400) in at least 10 fields of view in each section. The apoptosis index (AI) was calculated using the following formula: AI (%) = number of apoptotic cells/number of total cells x 100%.

**Cytokine measurement.** The concentration of tumor necrosis factor-α (TNF-α; cat. no. RK00027), interleukin (IL)-6 (cat. no. RK00008) and IL-1β (cat. no. RK00006) in lung tissue homogenate were measured using commercially available ELISA kits obtained from Abclonal Biotech Co., Ltd., Woburn, MA, USA) according to the manufacturer's protocol. The absorbance of each well was detected at 450 nm with a microplate reader. Each average value represents the values of triplicate experiments.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean and SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was utilized to perform statistical analysis. Statistical comparisons between multiple groups were performed using one-way analysis of variance followed by a bonferroni post-hoc. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of PHC on the PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> of T/HS rats.** As an evaluation index of gas exchange, PaO<sub>2</sub>/FiO<sub>2</sub> was measured to estimate the degree of lung injury. Significant decreases in PaO<sub>2</sub> (Fig. 1A) and the PaO<sub>2</sub>/FiO<sub>2</sub> ratio (Fig. 1B) were observed in T/HS rats compared with the sham group. However, when compared with the T/HS group, pre-treatment or treatment with PHC efficiently increased the PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> ratios in ALI rats (P<0.05).

**Effect of PHC on PMNs and BALF protein concentration in T/HS rats.** PMNs are the primary inflammatory cells present in many diseases of the lung, including ALI/ARDS (19).



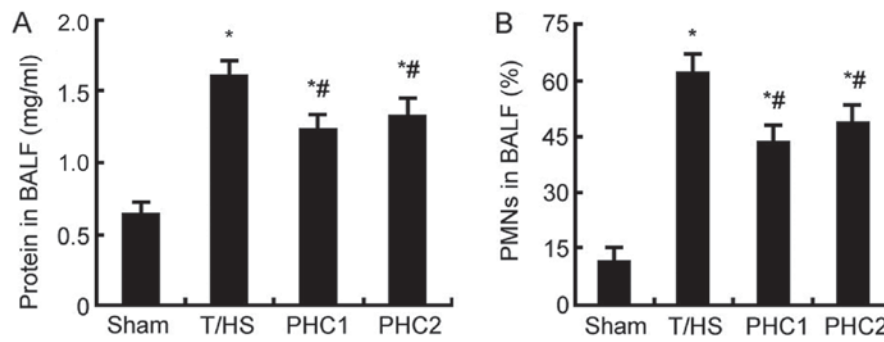


Figure 2. Effect of PHC on PMNs and protein concentration in BALF. The (A) protein concentration of and (B) the number of PMNs in BALF were assessed in the T/HS, PHC1 and PHC2 groups. The number of PMNs and the concentration of protein were decreased in the PHC1 and PHC2 groups compared with the T/HS group. Data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs. the Sham group; # $P < 0.05$  vs. the T/HS group. PHC, penehyclidine hydrochloride; PMN, polymorphonuclear neutrophils; BALF, bronchoalveolar lavage fluid; T/HS, blunt chest trauma and hemorrhagic shock.

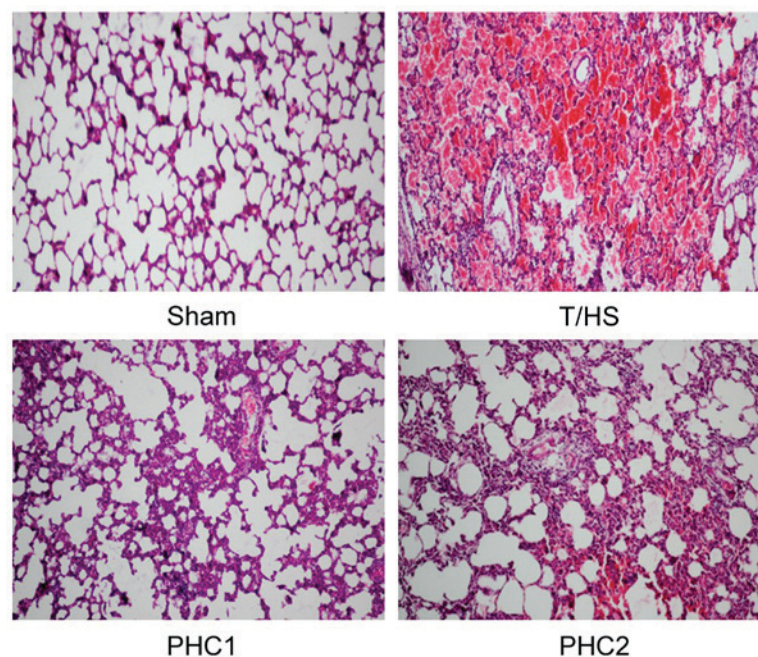


Figure 3. Effect of PHC on the lung histopathological changes observed in ALI rats. Hematoxylin and eosin staining revealed that severe hemorrhage and congestion, thickening of the alveolar wall, alveolar collapse and infiltration of alveoli with inflammatory cells was observed in the lung tissues of T/HS, PHC1 and PHC2 treated rats, compared with the sham group. However, compared with the T/HS group, lung injury was significantly ameliorated in the PHC1 and PHC2 groups. Original magnification,  $\times 100$ . PHC, penehyclidine hydrochloride; ALI, acute lung injury; T/HS, blunt chest trauma and hemorrhagic shock.

Furthermore, the concentration of protein in BALF is an indicator commonly used to detect pulmonary vascular permeability, which is an important characteristic of ALI/ARDS (20). When compared with the sham group, the concentration of protein (Fig. 2A) and the number of PMNs (Fig. 2B) in BALF were significantly increased 6 h following T/HS. However, PHC treatment prior to or following T/HS significantly reduced PMN infiltration and protein concentration compared with the T/HS group.

**Effect of PHC on the histopathological changes observed in T/HS rats.** To assess the effect of PHC on the histopathological changes observed in ALI rats, lung tissues from each group were subjected to H&E staining. As presented in Fig. 3, the sham group exhibited normal pulmonary histology with intact structures and clear pulmonary alveoli. By contrast, the tissue of the T/HS group exhibited serious damage, including severe

hemorrhage and congestion, alveolar wall thickening, alveolar collapse and inflammatory cell infiltration into alveoli. However, the administration of PHC ameliorated pulmonary histopathological changes in ALI rats.

**Effect of PHC on the expression of Fas, FasL, caspase-3 and caspase-8.** Western blotting was performed to detect the level of Fas, FasL, caspase-3 and caspase-8 proteins. The results revealed that, in response to T/HS, the protein expression of Fas, FasL, caspase-3 and caspase-8 were significantly increased; while PHC treatment administered prior to or following T/HS induction significantly inhibited this increase (Fig. 4).

**Effect of PHC on Fas and FasL protein expression in ALI lung tissue.** Immunohistochemistry assays were performed to detect the protein expression of Fas and FasL. The results revealed that, when compared with the sham group, T/HS

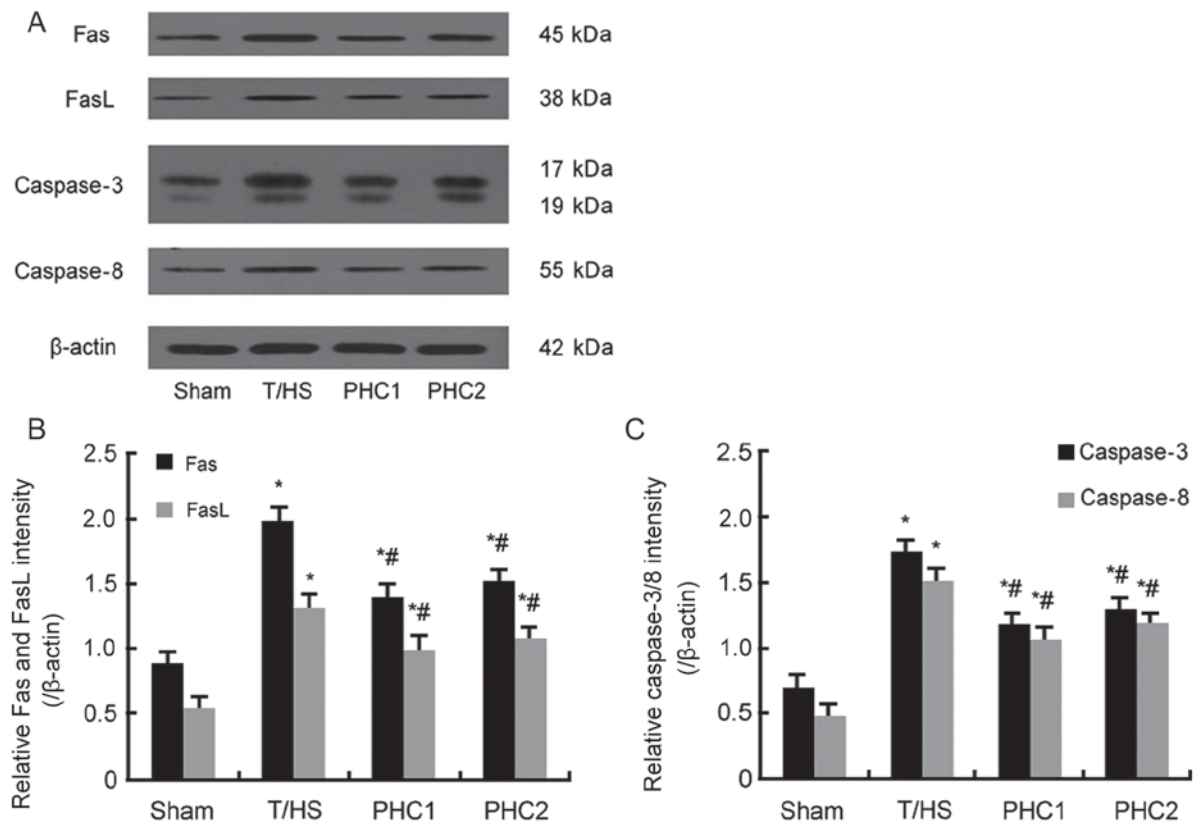


Figure 4. Effect of PHC on the expression of Fas, FasL, caspase-3 and caspase-8. (A) Western blotting was performed to assess protein levels of Fas, FasL, caspase-3 and caspase-8. (B) Statistical analysis of (B) Fas and FasL, and (C) caspase-3 and caspase-8. Increased levels of all proteins were observed in the lung tissue of T/HS, PHC1 and PHC2 treated rats when compared with the Sham group. However, protein levels were decreased in the PHC1 and PHC2 groups compared with the T/HS group. Data are expressed as mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs. the Sham group; # $P < 0.05$  vs. the T/HS group. PHC, penethylidine hydrochloride; FasL, Fas ligand; T/HS, blunt chest trauma and hemorrhagic shock.

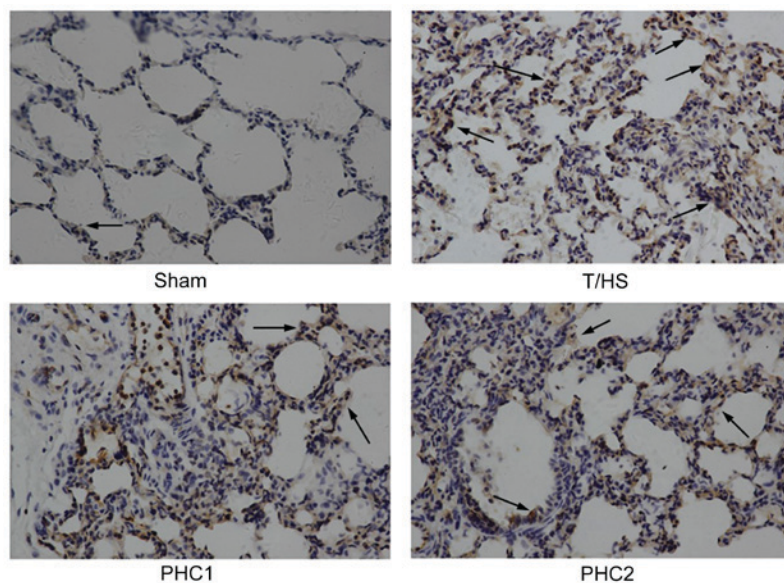


Figure 5. Effect of PHC on Fas protein expression in the lung alveolar area of rats. In the T/HS, PHC1 and PHC2 groups, the protein levels of Fas were markedly increased compared with the sham group. However, Fas protein expression was decreased in the PHC1 and PHC2 groups compared with the T/HS group. Black arrows indicate Fas protein expression. PHC, penethylidine hydrochloride; T/HS, blunt chest trauma and hemorrhagic shock.

induction markedly increased the protein levels of Fas (Fig. 5) and FasL (Fig. 6) in the alveolar area of the lung. Furthermore, the administration of PHC markedly reduced Fas and FasL levels compared with the T/HS group.

*Effect of PHC on the degree of apoptosis in ALI rat lung tissue.* As presented in Fig. 7, a small number of apoptotic cells were observed in the sham group (Fig. 7A), but apoptotic cells were significantly increased following T/HS



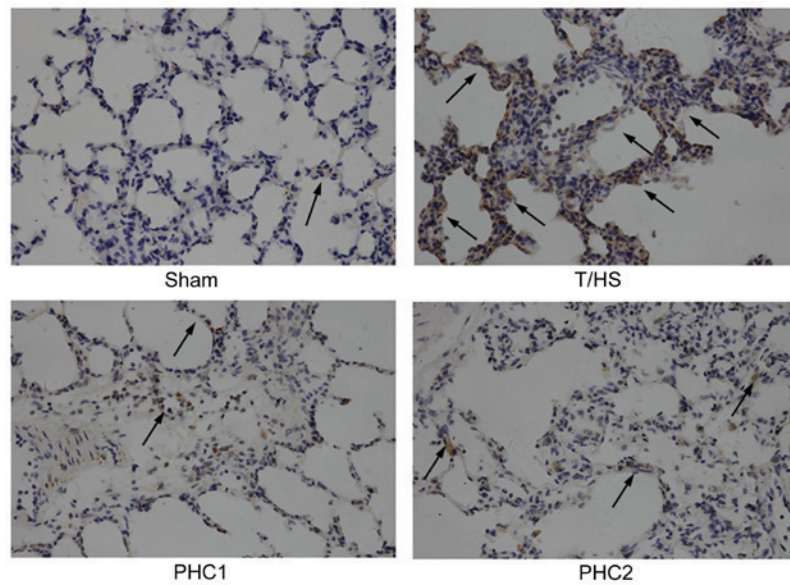


Figure 6. Effect of PHC on FasL protein expression in lung tissue. In the T/HS, PHC1 and PHC2 groups, the protein levels of FasL in the alveolar area of the lung were markedly increased compared with that the sham group. Fas protein levels were however decreased in the PHC1 and PHC2 groups when compared with the T/HS group. Black arrows indicate FasL protein expression. PHC, penethylidine hydrochloride; FasL, Fas ligand; T/HS, blunt chest trauma and hemorrhagic shock.

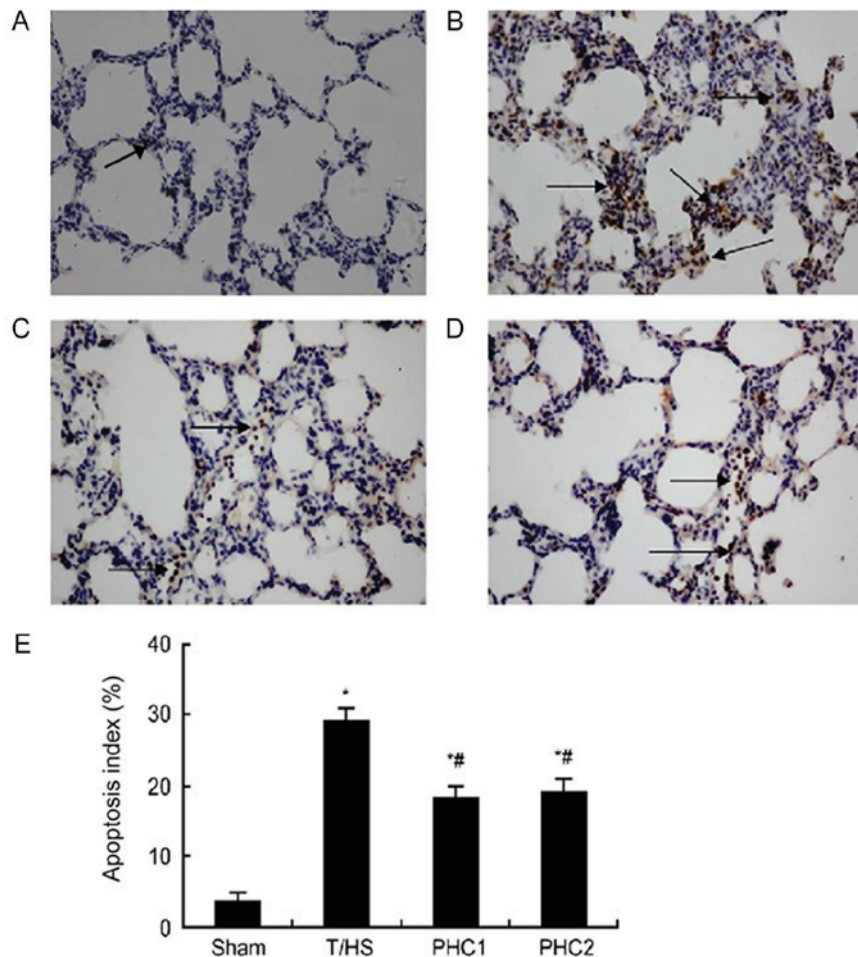


Figure 7. Effect of PHC on the degree of apoptosis in ALI rat lung tissue. A small number of apoptotic cells were observed in the (A) sham group. Compared with the sham group, apoptotic cells were significantly increased in the (B) T/HS, (C) PHC1 and (D) PHC2 groups. However, the number of apoptotic cells in the PHC1 and PHC2 groups was decreased compared with the T/HS group. The (E) apoptosis index of lung tissues in T/HS rats was significantly increased compared with the sham group and the apoptosis index was statistically decreased in the PHC1 and PHC2 groups compared with the T/HS group. Black arrows indicate apoptotic cells. Data are expressed as mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs. the Sham group; # $P < 0.05$  vs. the T/HS group. PHC, penethylidine hydrochloride; ALI, acute lung injury; T/HS, blunt chest trauma and hemorrhagic shock.

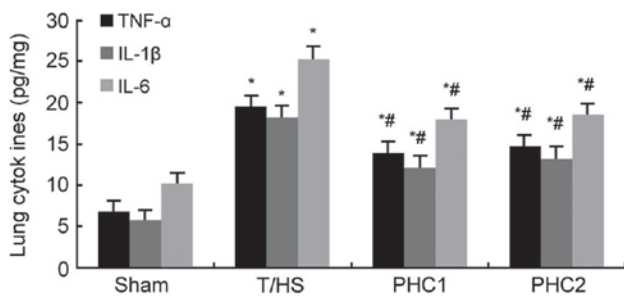


Figure 8. Effect of PHC on TNF- $\alpha$ , IL-6 and IL-1 $\beta$  levels in ALI rat lung tissue. Compared with the sham group, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in lung tissues were significantly increased in the T/HS, PHC1 and PHC2 groups. Compared with the T/HS group, the levels of these cytokines were significantly decreased in the PHC1 and PHC2 groups. Data are expressed as mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs. the Sham group; # $P < 0.05$  vs. the T/HS group. PHC, penicillin hydrochloride; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin; ALI, acute lung injury; T/HS, blunt chest trauma and hemorrhagic shock.

induction (Fig. 7B, C and D). Compared with the T/HS group, the number of apoptotic cells in the PHC1 and PHC2 groups was decreased (Fig. 7C and D). Concomitantly, the apoptosis index of T/HS rat lungs was significantly increased compared with the sham group and the apoptosis index was statistically decreased following the administration of PHC (Fig. 7E).

**Effect of PHC on TNF- $\alpha$ , IL-6 and IL-1 $\beta$  levels.** Lung tissues were collected 6 h following T/HS induction and the levels of cytokines within were measured using ELISA. The results demonstrated that the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly increased in T/HS rats compared with the sham group (Fig. 8). However, PHC treatment prior to or following T/HS induction significantly reduced the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 compared with the T/HS group.

## Discussion

ALI and ARDS induce a continuum of lung changes arising from a wide variety of lung injuries, causing high morbidity and mortality rates in intensive care units (21). An increasing number of studies have demonstrated the critical role of Fas/FasL activation in pulmonary epithelial cells (22,23). However, little is known about the exact mechanism that underlies this. The present study established a novel double-hit rat model of ALI (blunt trauma followed by hemorrhage shock), induced by T/HS, and determined the effect of PHC on the Fas/FasL signaling pathway by assessing pulmonary apoptosis, inflammation and lung damage. The results indicated that the T/HS-constructed ALI rat model significantly increased arterial hypoxemia, alveolar edema, leucocytosis in the interstitial capillaries and alveolar hemorrhage in histological assessments, which is consistent with a previous study (12). Additionally, it was revealed that PaO<sub>2</sub>/FiO<sub>2</sub> in T/HS rats was  $< 300$ .

It has been demonstrated that a multitude of pathways are involved in the regulation of apoptosis, including Fas/FasL (24). Gil *et al* (25) indicated that the Fas signaling pathway was associated with ALI severity in mice. In the present study, to determine the involvement of the Fas/FasL signaling pathway

in the mediation of lung tissue cell apoptosis, the lungs of rats with ALI induced by T/HS were assessed via TUNEL staining 6 h following T/HS. The results demonstrated that following T/HS challenge, the expression of Fas, FasL, caspase-3 and caspase-8 was significantly increased, and the BALF PMN count and protein concentration were markedly increased. The current study therefore hypothesized that Fas/FasL activation resulted in an increase of proinflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ) and apoptotic cells in the lungs during T/HS. In this regard, the activation of the Fas/FasL signaling pathway may not only induce apoptosis, but may also lead to the production and secretion of cytokines. Consistent with the findings of Weckbach *et al* (26), the combination of T/HS induced lung cell apoptosis, lung inflammation, subsequent PMN recruitment and disruption of the alveolocapillary barrier. Additionally, Serrao *et al* (27) demonstrated that PMNs induce the apoptosis of lung epithelial cells by upregulating FasL. Perl *et al* (28) also indicated that in the absence of PMNs, blunt chest trauma-induced ALI was mitigated. Therefore, lung cell apoptosis and lung inflammation have been identified as pathophysiologically relevant mechanisms in the development of ALI.

Multiple findings have revealed that PHC is effective in the treatment of ALI (29,30). Additional studies further indicate that PHC selectively blocks muscarinic acetylcholine (M) receptor M1, M3 and nicotinic acetylcholine receptors, with fewer M2 receptor-associated cardiovascular side effects than hyoscyamine (31). An increasing number of studies have indicated that PHC exhibits anti-apoptotic, anti-inflammatory and anti-oxidative stress effects under organ dysfunction (16,32,33). A previous study demonstrated that PHC exerted anti-inflammatory properties and protective effects during ALI via the inhibition of the toll-like receptor 4 signaling pathway (34). Furthermore, Cao *et al* (35) confirmed that PHC alleviated cerebral injury by inhibiting the p38 mitogen-activated protein kinase (MAPK) and caspase-3. Wang *et al* (36) also revealed that the administration of PHC in renal ischemia-reperfusion injured rats decreased the level of malondialdehyde and the expression of p38 MAPK, nuclear factor- $\kappa$ B and caspase-3 expression, and attenuated the reduction in superoxide dismutase activity. Wu *et al* (34) clarified that PHC significantly increased PaO<sub>2</sub>, pH, PaO<sub>2</sub>/FiO<sub>2</sub> and PaCO<sub>2</sub>, reduced IL-6 and IL-1 $\beta$  levels, and reduced pulmonary myeloperoxidase activity in an ALI model induced by lipopolysaccharide.

The results of the current study revealed that PHC treatment prior to or following T/HS induction attenuated lung damage by significantly improving pulmonary oxygenation and by decreasing BALF PMN count and protein concentration. Furthermore, the Fas/FasL signaling pathway, levels of cell apoptosis and the expression of proinflammatory cytokines, including TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , were inhibited following PHC treatment. Whether PHC improves the overall survival of rats with ALI was not assessed in the present study and as such, should be a subject of future experiments. Additionally, the exact mechanism of how PHC ameliorates ALI remains poorly understood, which warrants future investigation.

In conclusion, the present study indicates that the Fas/FasL signaling pathway may serve a pivotal role in the pathogenesis of lung injury following T/HS. PHC may also serve a protective

role in ALI by inhibiting the Fas/FasL signaling pathway. Therefore, PHC may be a potential agent for future treatment of inflammatory diseases, including ALI.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XW and XS designed the experiments. XW, QK and WD performed the experiments. WD and LZ analyzed experimental results. XW, QK and XS wrote the manuscript and critically revised it for important intellectual content. All authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Ethics approval was provided by the Medical Ethics Committee of Renmin Hospital of Wuhan University. All surgical procedures were performed in accordance with Wuhan University Animal Care and Use Committee.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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