

# Effects of Activated Protein C on Ventilator-Induced Lung Injury in Rats

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## Key Words

Bronchoalveolar lavage · Dimers · Macrophage inflammatory protein-2 · Plasminogen activator inhibitor-1 · Ventilator-induced lung injury

## Abstract

**Background:** Mechanical ventilation with a high tidal volume (VT) increases lung and systemic plasminogen activator inhibitor (PAI)-1 levels and alveolar fibrin deposition. Activated protein C (APC) may decrease PAI activity in endothelial cell-conditioned medium and thus enhance fibrinolysis. **Objectives:** The aims of this study were to test the hypothesis that APC can neutralize PAI-1 activity and improve lung function in an animal model of ventilator-induced lung injury. **Methods:** Rats were ventilated with a high-volume zero positive end-expiratory pressure (PEEP; HVZP) protocol by a volume-cycled ventilator for 2 h at a VT of 30 ml/kg, a respiratory rate of 25 breaths/min, and an FiO<sub>2</sub> of 0.21. Fifteen minutes before ventilation, the rats received intravenous APC (250 µg/kg, HVZP+APC group) or normal saline (vehicle; HVZP group). Another group that received no ventilation served as the control group. **Results:** Levels of arterial blood

gas tension were comparable between the two ventilation groups throughout the study period. Rats treated with the HVZP protocol exhibited significantly higher total protein and macrophage inflammatory protein-2 concentrations in bronchoalveolar lavage fluid (BALF) and higher lung PAI-1 mRNA expression and plasma active PAI-1 levels than did the control group. Administration of APC tended to reduce the BALF protein content and systemic PAI-1 activity but did not improve the lung histology in the HVZP+APC group. Plasma levels of D-dimers were comparable among the three study groups. **Conclusions:** These results suggest that APC administered at a higher dosage might improve lung function by reducing alveolar protein leakage and systemic coagulation.

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## Introduction

Over the last several decades, mechanical ventilation has successfully been used to support patients with acute respiratory failure. However, it has several potential disadvantages and complications regardless of the life-saving potential of this assistance [1]. Mechanical ventilation with high tidal volumes (VT) can damage the alveolar-capillary barrier and activates local and systemic inflammatory responses. This course is referred to as ventilator-induced lung injury (VILI) [2]. The spectrum of VILI includes disruption of endothelial and epithelial cells, and increases in endothelial and epithelial permeability and pulmonary and systemic inflammatory mediators [2–4]. Mechanisms implicated in VILI include alveolar epithelial and vascular endothelial cellular responses to mechanical stretching and inflammatory cell recruitment and activation [5, 6]. The physiological and histopathological features of VILI are indistinguishable from those of the diffuse alveolar damage of acute lung injury. Deposition of fibrin in the alveolar space and increased procoagulant activity of bronchoalveolar lavage fluid (BALF) are pathognomonic features of acute lung injury [7].

Additional evidence suggests that mechanical ventilation may influence pulmonary fibrin turnover in VILI [8, 9]. Alveolar fibrin deposition leads to surfactant dysfunction, poor gas exchange, decreased lung compliance, and increased ventilatory dependence, and accelerates the fibrotic process [10, 11]. The mechanisms that contribute to increased alveolar fibrin turnover are increased local tissue factor-mediated thrombin generation and depressed bronchoalveolar urokinase plasminogen activator-mediated fibrinolysis caused by increases in plasminogen activator inhibitors (PAIs) [12]. PAI-1, a fibrinolytic antiprotease, is the major plasminogen inactivator in the plasma and the primary inhibitor of tissue- and urokinase-type plasminogen activators resulting in decreased plasmin activity and fibrinolytic potential [13]. We found that mechanical ventilation with a high VT and no positive end-expiratory pressure (PEEP) increased lung and systemic PAI-1 levels and alveolar fibrin deposition [14]. Activated protein C (APC) decreases PAI activity in endothelial cell-conditioned medium and enhances fibrinolysis by inhibiting PAI-1 of the whole blood and serum [15, 16]. Administration of APC may suppress plasma PAI activity observed after thrombolysis in patients with acute myocardial infarction [17]. Therefore, we hypothesized that APC may inhibit PAI-1 activity and improve lung function in an animal model of VILI. The aims of this study were to investigate the effects of APC on local

and systemic fibrinolytic activities in VILI and to find a potential treatment modality against high-VT-induced lung injury.

## Materials and Methods

### *Animal Preparations*

This study was approved by the Animal Care and Use Committee of the Taipei Medical University and was performed with adult male Sprague-Dawley rats weighing 250–300 g. Rats were maintained on a 12-hour light-dark cycle with free access to food and water. Rats were intraperitoneally anesthetized with pentobarbital (50 mg/kg, Abbott, North Chicago, Ill., USA). Fifteen minutes before ventilation, rats received recombinant human APC [250 µg/kg i.v., drotrecogin α (activated); Xigris, Lilly, Giessen, Germany; n = 7; HVZP+APC group] or normal saline (HVZP group; n = 7) via the tail vein. A polyethylene tube catheter (PE-50, Becton Dickinson, Sparks, Md., USA) containing isotonic saline was placed in one femoral artery to sample the blood for gas analysis. A tracheostomy was performed, and a 14-gauge plastic cannula was inserted into the trachea. Animals were then ventilated with a high-VT zero PEEP (HVZP) protocol by a volume-cycled ventilator (Small Animal Ventilator, model SAR-830/AP; CWE, Ardmore, Pa., USA) for 2 h at a VT of 30 ml/kg, zero PEEP, a respiratory rate of 25 breaths/min, and FiO<sub>2</sub> of 0.21. Another group that received no ventilation served as the control group (n = 7). All animals were kept supine for the duration of the experiment, and arterial blood gases were measured with a blood gas analyzer (model 1620; Instrumentation Laboratories, Lexington, Mass., USA) at the beginning and every hour after randomization.

### *Bronchoalveolar Lavage*

After 2 h of ventilation, rats were killed with an intravenous injection of pentobarbital (100 mg/kg). Blood was collected from the femoral artery in citrated (0.109 M) vacutainer tubes, placed on ice, and spun at 4°C, and the resulting plasma was stored at -70°C until analyzed. The chest was opened and the lungs were removed intact from the animal with the tracheostomy tube in place. The right lung was ligated, and the left lung was lavaged with 2 ml 0.9% saline at 4°C which was washed in and out of the lungs three times and then recovered. This washing procedure was repeated two more times for each animal, with the three washes being pooled, and the total volume recorded. There were no differences in the total volume of saline infused or recovered after the lavage procedure among the three experimental groups. The right superior lobe was fixed in 10% buffered formalin and embedded in paraffin. An aliquot of the BALF from each animal was used to measure the total protein content with bovine serum albumin as the standard and macrophage inflammatory protein (MIP)-2 using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn., USA); values are expressed as milligrams per kilogram of body weight and picograms per milliliter of lavage fluid, respectively.

### *Lung PAI-1 mRNA Expression by Real-Time Polymerase Chain Reaction*

Lung tissue was ground into a powder in liquid nitrogen, and PAI-1 mRNA expression was measured using real-time PCR. Total

**Table 1.** Arterial blood PaO<sub>2</sub>, PaCO<sub>2</sub>, and pH at baseline (0 h) and after 1 and 2 h of ventilation

Group	n	pH	PaO <sub>2</sub> mm Hg	PaCO <sub>2</sub> mm Hg
Control	7			
0 h		7.41 ± 0.03	92 ± 8	43 ± 3
HVZP	7			
0 h		7.40 ± 0.06	93 ± 15	43 ± 10
1 h		7.62 ± 0.06	111 ± 6	21 ± 4
2 h		7.61 ± 0.03	110 ± 8	22 ± 2
HVZP+APC	7			
0 h		7.42 ± 0.04	95 ± 16	42 ± 3
1 h		7.61 ± 0.03	109 ± 11	22 ± 3
2 h		7.63 ± 0.02	101 ± 12	23 ± 3

RNA was extracted using the TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK). Reverse transcription was performed on 1 µg of RNA with oligo-dT primers and avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, Ind., USA). Primer sequences for the SYBR green real-time PCR included: PAI-1 sense (5'-ATGGCTCAGAACAACAAGTTCAAC-3') and antisense (5'-CAGTTCCAGGATGTCGTACTION-3'), and GAPDH rRNA sense (5'-ATGATTCTACCCACGGCAAG-3') and antisense primers (5'-CTGGAAGATGGTGATGGGTT-3'). Gene expression was quantitatively analyzed using the comparative CT (ΔCT) method, in which CT is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔCT method is the difference in the number of threshold cycles for a target (PAI-1) and an endogenous reference (the GAPDH rRNA housekeeping gene).

#### PAI-1 and the Dimer Assay

PAI-1 was assayed by a commercially available assay kit that measures active PAI-1 (Innovative Research, Southfield, Mich., USA) in the plasma and BALF. Fibrinolytic activity was assessed by measuring plasma concentrations of the D-dimer with an ELISA kit purchased from American Diagnostica (Stamford, Conn., USA).

#### Immunohistochemistry of PAI-1

Immunohistochemical staining for PAI-1 was performed on paraffin sections with immunoperoxidase visualization. After deparaffinization in xylene and rehydration in an alcohol series, sections were first preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum and 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity and nonspecific binding of the antibody before being incubated for 20 h at 4°C with a rabbit polyclonal antibody against rat PAI-1. Sections were then treated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, Calif., USA). This was followed by reaction with the reagents from an ABC kit (Avidin-Biotin Complex, Vector) according to the manufacturer's recommendations, and the reaction products were visualized by 3,3 diaminobenzidine and 0.003%

H<sub>2</sub>O<sub>2</sub> in 0.5 M Tris buffer (pH 7.6) before the sections were mounted on gelatin-coated slides using Permount (Fisher Scientific, Pittsburgh, Pa., USA). Sections for PAI-1 were mounted in glycerin gelatin and counterstained with hematoxylin. A minimum of four random lung fields of immunohistochemically stained sections per animal were captured with a digital camera and imported into the computerized image analysis system (Image-Pro Plus 5.1 for Windows). The automatic object counting and measuring process was used to quantify the immunoreactivity of the sections [14]. These generated a percentage of positively stained cells, and the value was expressed as a labeling index.

#### Histology

Specimens were embedded in paraffin, stained with hematoxylin and eosin, and examined by a pathologist who was blinded to the protocol and experimental groups. Lung injury was scored according to the following items: (1) alveolar congestion, (2) hemorrhage, (3) infiltration of neutrophils into the airspaces or the vessel wall, and (4) thickness of the alveolar wall [14]. Each item was graded according to a five-point scale: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage, and 4, maximal damage.

#### Statistical Analysis

The lung injury score data are given as medians and ranges, whereas other data are presented as means ± SD. Statistically significant differences were analyzed by ANOVA followed by Tukey's post hoc analysis. The Spearman test was used for correlation analyses of relationships between BALF protein contents and plasma active PAI-1 levels. Differences were considered significant at  $p < 0.05$ .

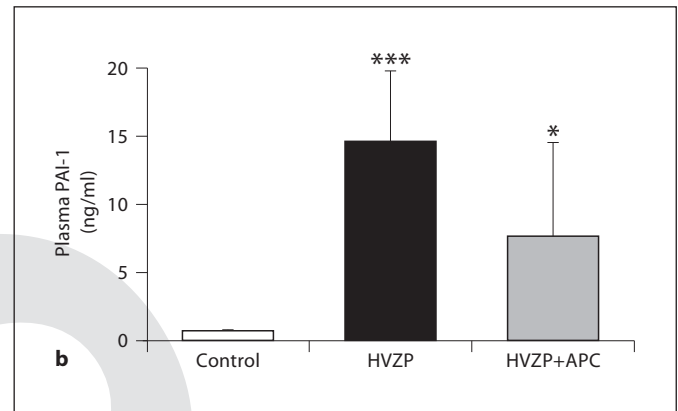
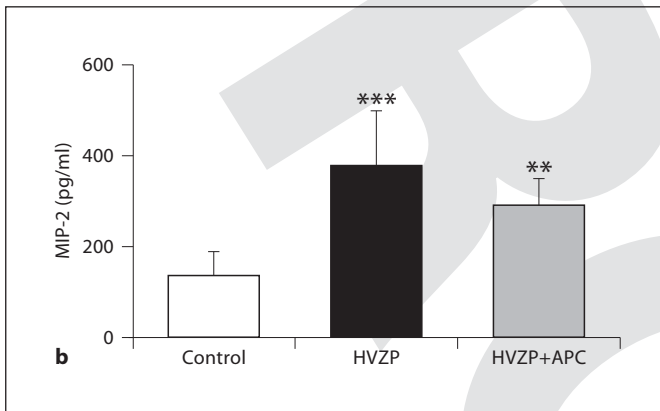
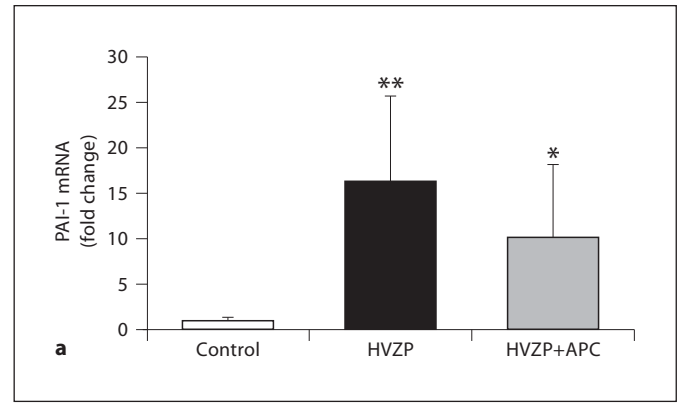
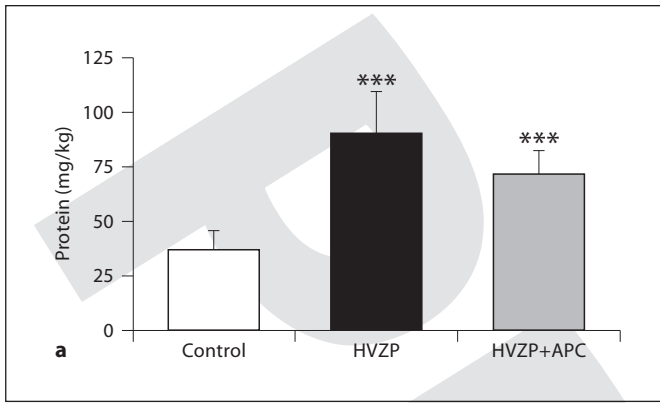
## Results

#### Gas Exchange

The arterial blood gas tensions were comparable among the three study groups before mechanical ventilation (table 1). Rats treated with HVZP ventilation exhibited a higher mean pH and lower mean carbon dioxide tension throughout the study period.

#### Total Protein, MIP-2, and PAI-1 in the BALF

Total protein contents recovered from the BALF were significantly higher in rats ventilated with the HVZP and HVZP+APC protocols than in the control group (fig. 1a). In the HVZP+APC group, we found a trend towards decreased BALF protein compared to the HVZP group ( $p = 0.059$ ). MIP-2 concentrations in the BALF increased after HVZP ventilation, and the values were approximately 2-fold higher in the HVZP group compared to the control group (fig. 1b). The addition of APC did not significantly decrease MIP-2 levels. PAI-1 activity was undetectable in all BALF samples from control animals and was barely detectable in HVZP-treated animals.



**Fig. 1.** Total protein and MIP-2 in BALF in the control (non-ventilated), high-V<sub>T</sub> zero PEEP (HVZP), and HVZP+APC groups. n = 7/group. **a** The BALF protein content was significantly higher in the HVZP and HVZP+APC groups compared to control rats (\*\*\*) p < 0.001 vs. the control group). **b** MIP-2 recovered from BALF was significantly higher in rats in the HVZP and HVZP+APC groups than in the control group (\*\* p < 0.01 and \*\*\* p < 0.001, vs. the control group).

**Fig. 2.** Lung PAI-1 mRNA expression and plasma active PAI-1 levels in the control, HVZP, and HVZP+APC groups. Treatment details are given in the legend to figure 1. **a** Rats treated with the HVZP and HVZP+APC protocols had significantly higher levels of lung PAI-1 mRNA expression compared to the control group (\* p < 0.05 and \*\* p < 0.01, vs. the control group). **b** Compared to the control group, rats treated with the HVZP and HVZP+APC protocols demonstrated significantly higher plasma PAI-1 levels (\* p < 0.05 and \*\*\* p < 0.001, vs. the control group).

#### Lung PAI-1 mRNA Expression and Plasma PAI-1 Levels

PAI-1 mRNA expression was significantly increased (~15-fold) in rats ventilated with the HVZP and HVZP+APC protocols in comparison to the control animals (fig. 2a). Compared to the control group, rats treated with HVZP ventilation demonstrated significantly higher plasma PAI-1 levels (fig. 2b). The HVZP+APC group tended to have lower PAI-1 levels than the HVZP group (p = 0.056).

#### Immunohistochemistry of PAI-1

PAI-1 immunoreactivity was mainly detected in airway epithelial and mesenchymal cells (fig. 3a–c), and the

immunoreactivity markedly increased in rats treated with HVZP compared to the control and HVZP+APC groups by quantitative analysis (fig. 3d).

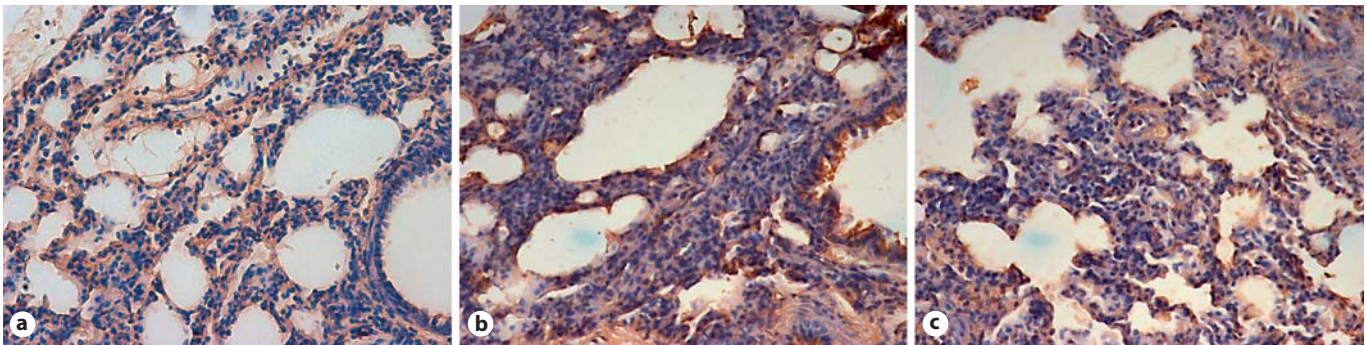
#### Plasma D-Dimers

Rats treated with the HVZP protocol exhibited non-significantly higher levels of plasma D-dimers compared to the control and HVZP+APC groups (fig. 4).

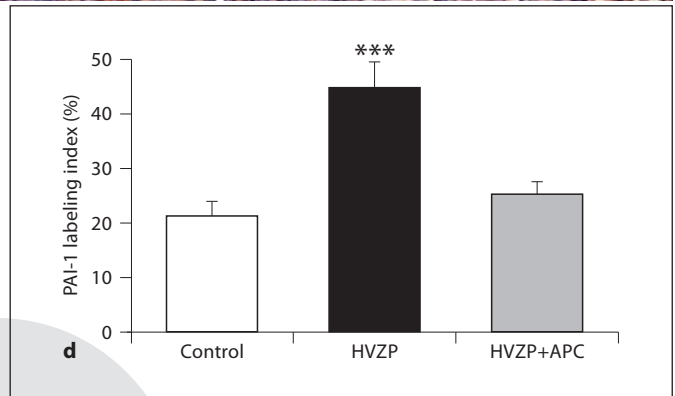
#### Histology

After 2 h of ventilation, the HVZP and HVZP+APC groups had significantly higher lung injury scores than the control group (table 2). Lung injury was characterized by alveolar congestion, hemorrhaging, and inflammato-





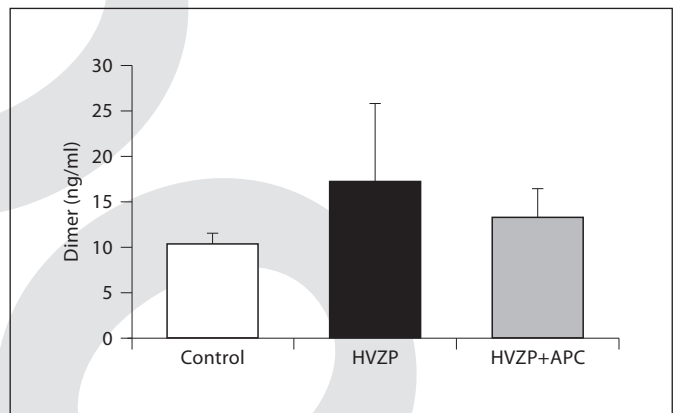
**Fig. 3.** Immunohistochemical staining for PAI-1 in the control (non-ventilated; **a**), HVZP (**b**), and HVZP+APC groups (**c**).  $\times 200$  (online version: brown = positive staining). **d** Quantitative analysis of PAI-1 immunoreactivity. PAI-1 immunoreactivities were mainly detected in airway epithelial and some mesenchymal cells, and the immunoreactivity markedly increased in rats treated with HVZP compared to the control (non-ventilated) and HVZP+APC groups (\*\* $p < 0.001$ ).



ry cell infiltration (fig. 5). These findings are consistent with changes in alveolar damage found with acute lung injury. The HVZP group showed patchy areas of hemorrhaging and thickened alveolar walls, and the HVZP+APC group showed less hemorrhaging and alveolar congestion. No major histological abnormalities were present in the control animals.

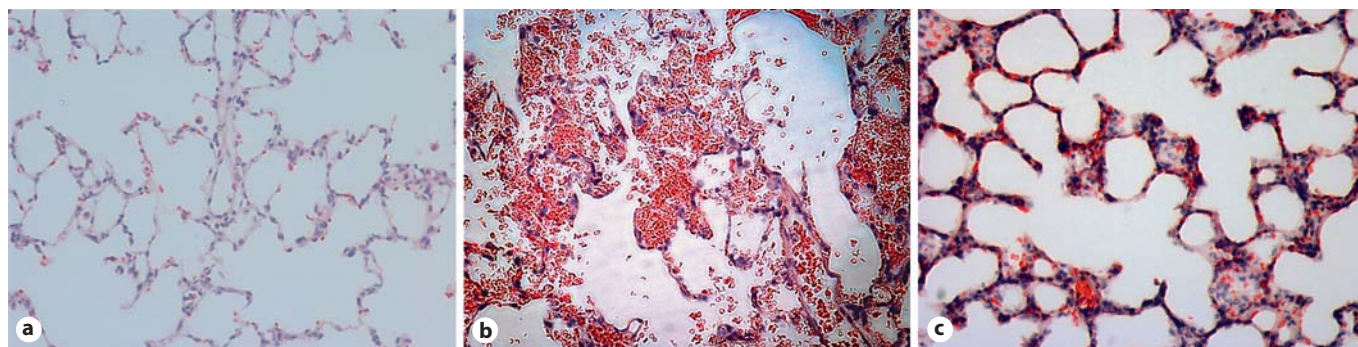
### Discussion

The main findings of this study are that high-VT ventilation is associated with increased lung capillary-alveolar leakage, BALF MIP-2 levels, lung PAI-1 mRNA expression, and plasma active PAI-1 levels. The findings of our in vivo lung injury model are consistent with alterations known to occur in VILI. These data indicate that high-VT ventilation may enhance local and systemic coagulation and suppress systemic fibrinolysis. The BALF protein content is a measure of capillary-alveolar protein leakage which is due to physical disruption of the plasma membrane integrity. In this study, we found that administration of APC did not significantly decrease the BALF protein content. These results are in contrast to the findings of Finigan et al. [18], who reported that APC treat-



**Fig. 4.** Plasma D-dimer in the control (non-ventilated), HVZP, and HVZP+APC groups. Rats treated with HVZP ventilation had nonsignificantly higher plasma D-dimer levels compared to the control and HVZP+ACP groups.

ment significantly decreased pulmonary leakage induced by mechanical ventilation when given either before or after initiation of ventilation. This discrepancy can possibly be explained by different APC dosages and different magnitudes of lung injury.



**Fig. 5.** Representative lung tissue photomicrographs ( $\times 200$ ). **a** Control group showing no major histological abnormalities. **b** HVZP group showing patchy areas of hemorrhaging and thickened alveolar walls. **c** HVZP+APC group showing less hemorrhaging and alveolar congestion.

**Table 2.** Lung injury scores (medians and ranges)

Treatment	n	Alveolar congestion	Hemorrhage	Neutrophil infiltration	Alveolar wall thickness	Lung injury score
Control	7	0 (0–1)	1 (0–2)	1 (0–1)	1 (1–2)	2 (1–3)
HVZP	7	3 (2–4)	3 (1–4)	2 (2–4)	2 (1–2)	11 (6–14)***
HVZP+APC	7	2 (2–4)	2 (2–4)	2 (2–4)	1 (1–2)	7 (7–14)***

\*\*\*  $p < 0.001$  vs. control.

In this study, although rats treated with HVZP ventilation exhibited lung tissue injury, arterial blood tension levels were comparable between the two study groups. These data suggest that monitoring of gas exchange is not a dependable indicator for the early recognition of VILI in previously healthy lungs. These results are supported by studies by Moriondo et al. [19] and Villar et al. [20], who found satisfactory results of arterial blood gas analysis in rats with high-VT-induced lung tissue injury.

Increased procoagulant activity of the BALF is a pathognomonic feature of acute lung injury [7, 21]. In this study, PAI-1 activity was undetectable in all BALF samples from control animals and barely detectable in HVZP-treated animals. We speculated that the difficulties in quantifying PAI-1 are because lung injury evolves rapidly and makes the timing of the analysis critical or the BAL procedure dilutes the alveolar contents. We also found that the total protein in the BALF was positively correlated with plasma levels of PAI-1 in all study animals ( $r = 0.695$ ,  $p < 0.001$ ). These results suggest that abnormalities in coagulation and fibrinolysis may play impor-

tant roles in the pathogenesis of VILI. Animal models of VILI showed that high-VT ventilation increases alveolar fibrin deposition and systemic PAI-1 activity, and lung-protective mechanical ventilation decreases BALF levels of PAI-1, attenuates coagulation, and enhances fibrinolysis [14, 22]. APC exerts its anticoagulant activity by inactivating procoagulant factors Va and VIIa and promotes fibrinolysis by inactivating PAI-1 [23]. Recombinant APC was shown to significantly reduce mortality in patients with severe sepsis [24]. Theoretical considerations suggest that anticoagulant therapy with APC might benefit animals with VILI. APC was shown not only to be an anticoagulant but also to have anti-inflammatory properties [25, 26]. The distinctive combination of anticoagulant and anti-inflammatory properties of APC has made it an important natural anticoagulant. However, APC did not improve lung function, although APC treatment tended to reduce systemic PAI-1 activity in this animal model of VILI.

D-dimers are derived from the degradation of cross-linked fibrin polymers, which come from clots, not free-

ly circulating fibrin or fibrinogen. Elevated levels of D-dimers indicate increases in blood coagulation and turnover of cross-linked intravascular fibrin activation [27]. In this murine model of VILI, we found that high-VT ventilation increased plasma levels of D-dimers, and APC treatment did not significantly decrease plasma D-dimer levels. These results suggest that APC treatment at this dosage cannot inhibit fibrin formation or activate fibrinolysis.

APC is a serine protease with potent anti-inflammatory actions in addition to its anticoagulation properties [28]. The anti-inflammatory activity of APC depends on its ability to suppress the secretion of several cytokines [29]. MIP-2 is associated with leukocyte migration and activation, and was found to be closely associated with them in animal models of pneumonia and VILI [30, 31]. Intravenous APC significantly decreased BALF levels of MIP-2 in leukocyte elastase-induced lung injury in mice [32]. In this study, plasma levels of active PAI-1 tended to

significantly decrease, and BALF levels of MIP-2 were comparable to those with APC treatment. These changes imply that the anticoagulant effect and anti-inflammatory activity of APC are independent of each other. The major limitation of this study is a lack of a dose-response analysis. There are potential beneficial effects at the APC dose used, and the possibility exists that higher doses may be more efficacious in reducing VILI.

In conclusion, systemic coagulation-fibrinolysis abnormalities induced by high-VT ventilation were indicated by increased pulmonary PAI-1 mRNA expression and plasma levels of PAI-1. Intravenous administration of APC tended to be associated with decreased alveolar protein leakage and plasma levels of PAI-1 and with comparable BALF levels of MIP-2 but could not improve oxygenation or the lung histology. We speculate that APC administered at a higher dosage might improve lung function by reducing alveolar protein leakage and systemic coagulation.

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