

Ventilator–induced coagulopathy in experimental *Streptococcus pneumoniae* pneumonia

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Short title: ventilator-induced coagulopathy in pneumonia

Abstract

Pneumonia, the main cause of acute lung injury, is characterized by a local proinflammatory response and coagulopathy. Mechanical ventilation (MV) is often required. However, MV can lead to additional injury so-called ventilator-induced lung injury (VILI). Therefore, we investigated the effect of VILI on alveolar fibrin turnover in *Streptococcus pneumoniae* pneumonia.

Pneumonia was induced in rats, followed 48 hours later by either lung-protective MV (lower tidal volumes (V_T) and positive end-expiratory pressure; LV_T -PEEP) or MV causing VILI (high V_T , zero-PEEP; HV_T -ZEEP) for 3 hours. Non-ventilated pneumonia rats and healthy rats served as controls. Thrombin anti-thrombin complexes (TATc), as a measure for coagulation, and plasminogen activator activity, as a measure of fibrinolysis, were determined in bronchoalveolar lavage fluid (BALF) and serum.

Pneumonia was characterized by local (BALF) activation of coagulation, resulting in elevated TATc-levels and attenuation of fibrinolysis compared to healthy controls. LV_T -PEEP did not influence alveolar coagulation nor fibrinolysis. HV_T -ZEEP did intensify the local procoagulant response: TATc-levels rose significantly and levels of the main inhibitor of fibrinolysis, plasminogen activator inhibitor (PAI)-1, increased significantly. HV_T -ZEEP also resulted in systemic elevation of TATc compared to LV_T -PEEP.

MV causing VILI increases pulmonary coagulopathy in an animal model of *S. pneumoniae* pneumonia and results in systemic coagulopathy.

Keywords: ARDS, biotrauma, coagulation, mechanical ventilation, pneumonia

Funding: Jack J Haitzma is a recipient of the Eli Lilly–University of Toronto, Critical Care Medicine Fellowship – Marcus J Schultz is supported by a personal grant from the Netherlands Organization for Health Research and Development (ZonMW); NWO-VENI grant 2004 [project number 016.056.001] – Jan W. Kuiper is supported by a stipend from the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences, The Netherlands – Haibo Zhang and Arthur S. Slutsky are supported by the Canadian Institutes of Health Research (CIHR).

Introduction

Community-acquired pneumonia is the most frequent cause of acute lung injury (ALI) or its more severe form acute respiratory distress syndrome (ARDS), often requiring mechanical ventilation (MV) (1). Although mechanical ventilation provides essential life support, it can also worsen lung injury; so-called ventilator-induced lung injury (VILI). One large multicenter trial established the importance of VILI by demonstrating that ventilation with lower tidal volumes (V_T) versus traditional V_T (6 vs. 12 mL/kg) improves survival (2). The spectrum of VILI also encompasses increases in pulmonary and systemic inflammatory mediators so called biotrauma (3-6), which has been linked to multiple organ failure (7).

Pulmonary inflammation associated with pneumonia is characterized by local generation of proinflammatory mediators and a procoagulant shift of the alveolar hemostatic balance (8). The latter is the result of activation of coagulation on the one hand, causing alveolar fibrin production, and attenuation of fibrin breakdown on the other (8, 9). Disturbances in alveolar fibrin turnover have been demonstrated in patients with pneumonia (10, 11) and ALI/ARDS (11).

Recently, we demonstrated that in models of VILI, local pulmonary fibrinolysis is suppressed during injurious mechanical ventilation, so-called ventilator-associated coagulopathy (12, 13). However, the models used were iatrogenic in nature, a single exposure to LPS (13) or fibrin deposits caused by human plasma without inflammation (12) and no systemic analysis were performed. It is unknown whether mechanical ventilation affects the pulmonary coagulopathy in pneumonia and if this can lead to systemic changes. In the current study we determined the effect of VILI on pulmonary

and systemic coagulopathy in a clinically relevant model of *Streptococcus pneumoniae* pneumonia.

Materials and methods

All studies were approved by the animal care committee at St. Michael's Hospital in accordance with Canadian Council of Animal Care guidelines. A total of 57 male Sprague–Dawley rats (weight 240 – 300 g, Charles River, Quebec, Canada) were included in this study.

Induction of pneumonia

As described before (14), *S. pneumoniae* pneumonia was induced by intratracheal instillation of live bacteria in 51 rats. In short, $\sim 5 \times 10^6$ CFU *S. pneumoniae* serotype 3 (ATCC 6303; Manassas, VA) per rat was aerosolized intratracheally using a trans–oral miniature nebulizer (Penn-Century, Philadelphia, PA, USA) (15) under light anesthesia (65% nitrous oxide/33% oxygen/2% isoflurane). Animals were allowed to recover from anesthesia and returned to their cages with food and water *ad libidum*. Rats were monitored every 8 hours and received supplemental fluids every 24 hrs by intraperitoneal injection (30 ml/kg of lactated Ringers solution). Fifteen non–ventilated rats with pneumonia and 6 non–ventilated rats without pneumonia served as controls (non–ventilated pneumonia controls and healthy controls, respectively).

Experimental protocol.

Forty–eight hours after intratracheal challenge with bacteria, animals were randomized (block randomization) to one of the two ventilation strategies or no ventilation. Thirty rats were anesthetized with an intraperitoneal injection of 100 mg/kg of ketamine (Ketalean, 100mg/ml, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada)

and 6 mg/kg of xylazine (Rompun 20mg/ml, Bayer Inc. Toronto, Ontario, Canada). A sterile metal cannula was inserted into the trachea and a polyethylene catheter was inserted into the carotid artery. Anesthesia was maintained by continuous intravenous infusion of ketamine (15 mg/kg/hour), xylazine (3 mg/kg/hour); paralysis was achieved by infusing pancuronium (0.35 mg/kg/hour) – medications were infused via a tail vein catheter. Animals were subsequently ventilated in the volume-controlled mode (Servo Ventilator 300, Maquet, Solna, Sweden), at a fractional inspired oxygen concentration (FiO_2) of 0.4, inspiration to expiration ratio of 1:2. V_T was initially set at 6 ml/kg body weight with positive end-expiratory pressure (PEEP) of 5 cm H_2O .

After a 10-minute stabilization period rats were randomized to either a lung protective strategy using lower V_T and PEEP ($\text{LV}_T\text{-PEEP}$; $n=15$) or a strategy causing VILI with higher V_T (12 ml/kg body weight) and zero end-expiratory pressure ($\text{HV}_T\text{-ZEEP}$; $n=15$) as previously reported (16). Settings were based on current knowledge that both increased V_T and lack of PEEP are causative parts of VILI (7, 17), and to keep mean airway pressures between the groups similar and hence minimizing the effect on mean arterial blood pressure (18). Animals were ventilated for 3 hours. Blood pressure was continuously monitored through the intra-arterial catheter. Blood samples of 100 μl were taken hourly and cultured undiluted onto sheep-blood agar plates, incubated and counted after 24 hours. Blood gas determinations were performed hourly, using a pH/blood gas analyzer (Ciba-Corning Model 248 blood gas analyzer; Corning Medical, Melfield, MA). Body temperature was maintained at 37°C using a heating pad. At the end of the experiment all animals were sacrificed by exsanguination.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed with saline 30 ml/kg body weight, two times and pooled. The number of viable bacteria in BALF was determined by plating 10-fold dilutions on sheep–blood agar plates; plates were incubated at 37°C in 5% CO₂ and counted after 24 hours. BALF was centrifuged at 4°C at 400 x g for 10 minutes, supernatant was snap-frozen on liquid nitrogen and stored at –80°C until further analysis.

Histology

In 3 animals of each group, lungs were taken for histopathology. Lungs were removed en bloc without BAL and fixed in 4% paraformaldehyde at a mean pressure of 10 cm H₂O. The specimens were embedded in paraffin, sectioned in tissue blocks from all lobes and stained with hematoxylin and eosin. The analyzing pathologist was not informed about the study purpose, blinded for the samples and only asked to score for lung injury. Scoring was done on: alveolar collapse, alveolar hemorrhage, perivascular edema, polymorphonuclear leukocytes infiltration, alveolar membranes and alveolar edema in a range of none, mild, moderate and severe (score 0, 1, 2, or 3, respectively) for descriptive purposes as previously described (19).

Coagulation and fibrinolysis

Levels of thrombin–antithrombin (TATc), plasminogen activator inhibitor (PAI)–1, and D–dimers were measured by ELISA in accordance with the manufacturers instructions (TATc, Behringwerke; PAI-1, Biopool; and Asserachrom D-dimer, Diagnostica Stago, Asnières-sur-Seine, France) in BALF–supernatants. Plasminogen activator activity

(PAA) and levels of antithrombin (AT) were measured using an amidolytical assay (20). Measurements were done simultaneously in BALF and plasma.

Cytokines

Concentrations of tumor necrosis factor- α (TNF- α) and interleukin-6 (Il-6) in BALF-supernatants and plasma were measured by using rat specific ELISA kits (BioSource, Camarillo, CA) in accordance with the manufacturers instructions.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 4. Because the primary objective of this study was the effect of different ventilation strategies on pneumonia, only the 3 pneumonia groups were compared (HV_T-ZEEP, LV_T-PEEP and non-ventilated pneumonia group). Inter-group differences were analyzed with analysis of variance (ANOVA). If ANOVA resulted in $p < 0.05$ a Bonferroni post-hoc test was performed. A $p < 0.05$ was considered statistically significant.

Results

Pneumonia

After intratracheal challenge with *S. pneumoniae* all animals developed severe pneumonia, resulting in the death of 6 of the infected animals (12%). The severity of the pneumonia was also evident by both macroscopic (a minimum of 25% of the lungs was visually affected at autopsy) as well microscopic histological examinations.

Microbiological examinations

There was no difference in the number of bacteria in BALF between non-ventilated pneumonia rats, and mechanically ventilated rats (Figure 1). Also, there was no difference in the number of bacteria in BALF from animals ventilated with the LV_T-PEEP strategy and animals subjected to HV_T-ZEEP. Similarly, the number of animals with bacteremia was not different: 8 out of 15 rats ventilated with the LV_T-PEEP strategy, and 7 out of 15 rats with HV_T-ZEEP showed positive blood cultures.

Gas exchange, lung elastance, mean arterial pressure

There were no differences in blood pressure during the period of MV (Figure 2). There were no differences in oxygenation in either MV group over time (Figure 2). All ventilated animals with pneumonia required intravenous fluid administration at the start of MV because of hypotension, but no differences existed between the two MV-groups (HV_T-ZEEP 25 ± 5 ml and LV_T-PEEP 24 ± 5 ml).

Pulmonary coagulation

S. pneumoniae pneumonia caused local activation of coagulation: BALF-levels of TATc increased from 0.9 ± 0.2 ng/ml in healthy rats to 4.0 ± 0.5 ng/ml in non-ventilated pneumonia rats (Figure 3). While LV_T-PEEP did not alter BALF-levels of TATc (4.0 ± 0.5 ng/ml), HV_T-ZEEP resulted in a significant increase of BALF-levels of TATc to 5.5 ± 0.7 ng/ml ($p < 0.001$ versus LV_T-PEEP). BALF-levels of the endogenous anticoagulant AT were highest in the healthy animals (20.0 ± 4.7 IU/ml) and were depressed in all pneumonia groups. Although BALF-levels of AT were lowest in the HV_T-ZEEP group, differences with LV_T-PEEP rats did not reach statistical significance.

Pulmonary fibrinolysis

PAA-levels in BALF were highest in healthy rats ($99.5 \pm 7.0\%$); pneumonia led to a decrease ($71.5 \pm 10.3\%$) (Figure 4). LV_T-PEEP did not alter PAA-levels in BALF ($71.4 \pm 7.0\%$), while injurious ventilation further decreased PAA-levels ($61.4 \pm 7.5\%$). BALF-levels of D-dimers were increased in all rats with pneumonia compared to healthy controls (296 ± 44 versus 13 ± 3 ng/ml). There was no effect of either MV strategy on BALF-levels or D-dimers. BALF-levels of PAI-1, the main inhibitor of fibrinolysis, were higher in non-ventilated pneumonia rats as compared to healthy animals (11.1 ± 1.7 ng/ml versus 1.4 ± 0.5 ng/ml). While LV_T-PEEP did not alter PAI-1 levels in BALF (11.6 ± 1.7 ng/ml), HV_T-ZEEP significantly increased PAI-1 levels (15.5 ± 1.7 ng/ml; $p < 0.001$ versus LV_T-PEEP).

Systemic coagulation and fibrinolysis

Activation of coagulation was also observed in plasma of non-ventilated pneumonia controls compared with healthy rats (Figure 4) (12.7 ± 1.0 ng/ml versus 4.7 ± 0.7 ng/ml). HV_T-ZEEP increased TATc in plasma (15.3 ± 2.0 ng/ml; $p < 0.05$ versus LV_T-PEEP and infected controls), while LV_T-PEEP did not alter systemic TATc-levels (12.5 ± 1.8 ng/ml). Systemic PAA-levels were significantly higher in healthy control animals as compared to non-ventilated pneumonia rats ($p < 0.05$); this was not altered by the MV-strategy.

Cytokines

IL-6 levels were increased both in BALF (1.03 ± 0.05 ng/ml versus 0.19 ± 0.01 ng/ml) and plasma (9.4 ± 2.2 ng/ml versus 0.15 ± 0.01 ng/ml) of non-ventilated pneumonia controls compared with healthy rats (Figure 5). HV_T-ZEEP further increased IL-6 levels in BALF (1.69 ± 0.31 ng/ml; $p < 0.05$ versus infected controls), while LV_T-PEEP did not alter BALF IL-6 levels. Ventilation did not affect systemic IL-6 levels.

TNF was increased in BALF of non-ventilated pneumonia controls compared with healthy rats (355 ± 72 pg/ml versus 51 ± 4 pg/ml) (Figure 5), LV_T-PEEP did not change TNF levels (388 ± 68 pg/ml) and HV_T-ZEEP resulted in the highest levels (632 ± 141 pg/ml) although this was not significantly different from other groups. Systemic TNF levels were higher in non-ventilated pneumonia rats as compared healthy control animals (367 ± 90 pg/ml versus 52 ± 5 pg/ml); this was not altered by the MV-strategy.

Histopathology

Histological analysis clearly showed pneumonia in all infected animals (Figure 6). HV_T-ZEEP resulted in increased infiltration of neutrophils and alveolar collapse, which was significantly higher compared to LV_T-PEEP (N=3 per group).

Discussion

The main finding of this study is that in lungs infected with *S. Pneumoniae*, HV_T–ZEEP promotes activation of coagulation and attenuation of fibrinolysis, while LV_T–PEEP leaves the pro–coagulant state in pneumonia unaltered. As far as we know, this study is among the first to show that MV can aggravate pulmonary coagulopathy in pneumonia. Importantly, these hemostatic changes were also found in the systemic compartment. Of note, differences in coagulation activation and fibrinolysis attenuation could not be explained by differences in numbers of bacteria present in the lung. Indeed, bacterial burden was not different after 3 hours of MV in the two MV–groups in our experiment. In addition, no differences in oxygenation and systemic hemodynamics were found during the course of MV.

In patients with established community–acquired or ventilator–associated pneumonia (11, 20), and in intubated and mechanically ventilated patients developing pulmonary infection (8, 10), there are procoagulant changes in the pulmonary compartment. Concomitant depressed levels of endogenous anticoagulant proteins and depressed fibrinolytic activity is also observed, and the combination of these changes leads to alveolar fibrin depositions. The findings in our model of *S. pneumoniae* pneumonia closely resemble those found in clinical studies. Similar to patients with pneumonia, coagulation was activated and fibrinolysis was attenuated in the pulmonary compartment of animals after challenge with bacteria. Also, levels of AT were lower after induction of pneumonia. Therefore, we consider our model of interest for pre–clinical studies on ventilator–induced coagulopathy, at least in the setting of pneumonia. Although animal models will never be able to simulate the complex diseases in patients and the time of

ventilation is also shorter than those in patients (3 hours versus days), the observation that even a short period of injurious ventilation in pneumonia causes coagulopathy emphasizes the consequences of injurious ventilation in general and specifically in high-risk groups such as pneumonia. It remains speculative whether our model has similarities with ALI/ARDS, although the findings in the pulmonary compartment in patients with ALI/ARDS closely resemble those found in patients with pneumonia (11).

We specifically chose *S. pneumonia* as the causative pathogen in our experimental setup, because community-acquired pneumonia is most often caused by this pathogen which is responsible for more than 500,000 cases of lower respiratory tract infection in the United States each year (21). Ventilator-associated pneumonia is commonly caused by *Pseudomonas* species and other Gram-negative bacteria (22). Similar changes in alveolar fibrin turnover, however, have been found in patients suffering from ventilator-associated pneumonia (10, 11). Furthermore, in models of *P. aeruginosa* pneumonia comparable procoagulant changes in BALF have been described (23). Of importance, in patients with ventilator-associated pneumonia it was recently shown that non-survivors showed significantly higher BALF-levels of TATc and PAI-1 than survivors (24) and increased PAI-1 levels also correlated with mortality and adverse clinical outcome in patients from the ARDS-network study (25). Similarly higher systemic PAI-1 levels are associated with poor prognosis in septic patients (26). Although the direct link between TATc and PAI-1 levels and mortality needs to be proven first, these data are very suggestive that MV strategies that promote coagulation and attenuate fibrinolysis may worsen prognosis.

MV alone has been demonstrated to affect pulmonary coagulation in other pre-clinical animal models, as well as in one clinical study (12, 13, 27). We recently showed in a rat model of MV that the use of increasing V_T , but with similar PEEP levels, caused a shift in alveolar fibrin turnover, mainly due to higher levels of PAI-1 in the lungs of these animals (12, 13). In addition, we recently reported on patients without pre-existing lung injury who were intubated and mechanically ventilated because of elective surgery (27). In these patients 5 hours of MV with traditional V_T (12 ml/kg ideal body weight) and no PEEP caused activation of pulmonary coagulation, as reflected by a marked increase in TATc-levels in BALF, while lung protective MV (using V_T of 6 ml/kg predicted body weight and 10 cm H₂O PEEP) did not cause a rise in TATc-levels (27). Based on these findings we suggest that coagulopathy may be an intrinsic component of ventilator-induced lung injury (or its clinical counterpart ventilator-associated lung injury).

Insights into the pathophysiology of VILI comes from animal studies which demonstrated that MV with larger V_T rapidly results in pulmonary changes that mimic ARDS (28). Injurious MV-settings resulted in recruitment and activation of inflammatory cells (29), local production of inflammatory mediators (e.g., cytokines) (30), and leakage of such mediators into the systemic circulation (4). Ranieri *et al.* confirmed a reduction in bronchoalveolar lavage fluid and systemic concentrations of inflammatory mediators during lung protective MV compared to conventional mechanical ventilation in a clinical trial (5, 31). IL-6 and TNF both were increased in all the pneumonia animals, however only VILI increased IL-6 in the lung. TNF an early release cytokine with a peak response a 1 – 2 hours was not increased, which corroborates previous findings (32). In a recent study using gene profiling, IL-6 was

shown to be one of the key cytokines to be affected during VILI while no effect was seen on TNF which is similar to our observations (33).

Although many studies have showed impairment of oxygenation to be a hallmark of lung injury, in our model gas exchange was not impaired similar to previous results (34). Nor did the oxygenation change during the period of ventilation which was most likely due to the tidal volume used in our model (12 ml/kg) which is considerably lower than other animal studies (with tidal volumes up to 45 ml/kg). Finally, improved gas exchange, with higher tidal volumes of 12 ml/kg, did not improve outcome in patients with ALI (2).

The current study has several limitations: we ventilated animals with *S. pneumoniae* pneumonia and can only demonstrate the additional effect of ventilation on pre-existing lung injury (coagulopathy) in a single model of pulmonary ALI. Furthermore, our applied ventilator strategies cannot separate the individual effects of either V_T or PEEP on the observed ventilator-induced coagulopathy. These limitations should be addressed in future studies.

In conclusion, injurious ventilation settings promote pro-coagulant changes and attenuates fibrinolysis in a rat model of pneumonia. These changes are not restricted to the site of inflammation, since changes in fibrin turnover were also found in the systemic compartment with injurious ventilation settings.

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Figure legends

Figure 1

Schematic representation of the study design

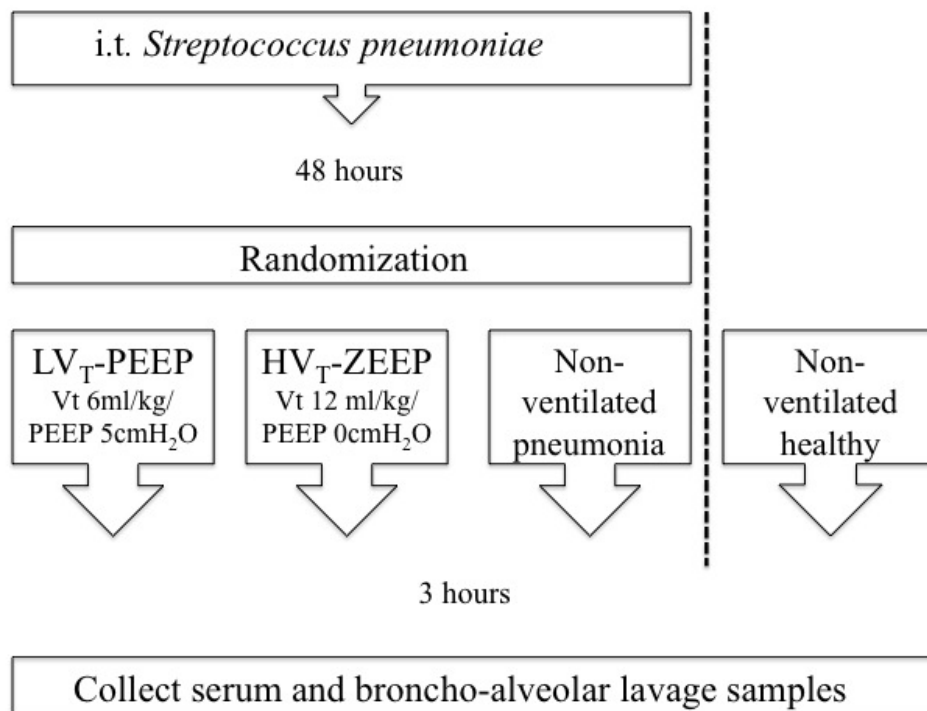


Figure 2

Number of colony forming units (CFU/ml) in bronchoalveolar lavage at the end of the experiment in the three groups with pneumonia. Infected; control group with *S. pneumoniae* pneumonia not ventilated (open diamonds), LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation (closed triangles), HV_T-ZEEP; group with pneumonia and VILI (inverted closed triangles).

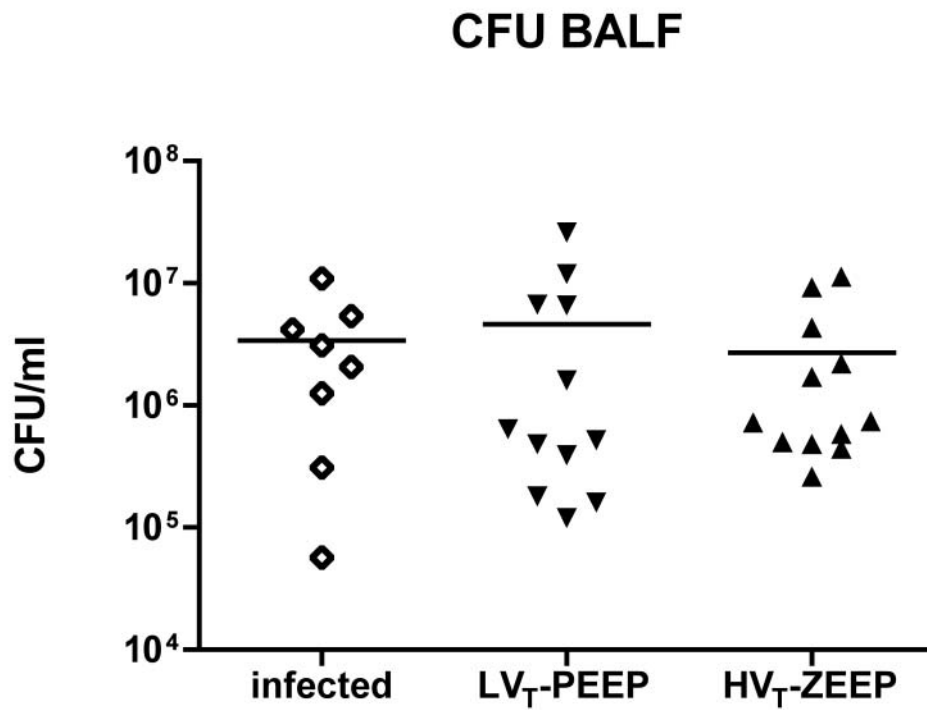
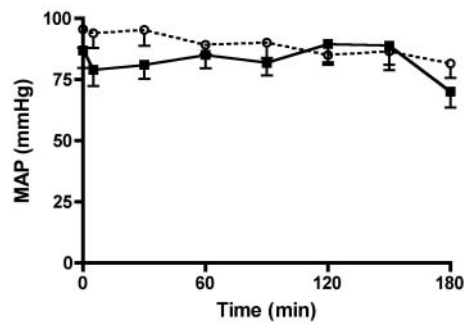
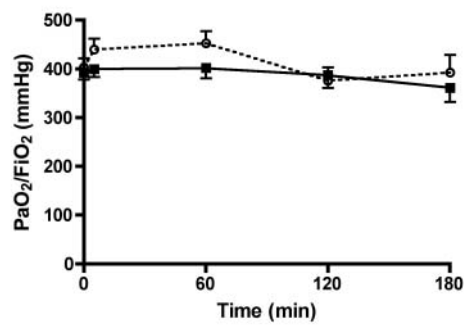


Figure 3

A) Mean arterial blood pressure overtime in the two ventilated pneumoniae groups. B) Mean arterial oxygenation ($\text{PaO}_2/\text{FiO}_2$) overtime in the two ventilated pneumoniae groups. LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation (open circles), HV_T-ZEEP; group with pneumonia and VILI (closed squares).

A**B****Figure 4**

Effect of pneumonia alone and mechanical ventilation and pneumonia on thrombin-anti-thrombin complex (TATc), antithrombin (AT), plasminogen activator inhibitor type 1 antigen (PAI-1), plasminogen activator activity (PAA), and D-dimers in bronchoalveolar lavage fluid. Non-infect; control group without pneumonia and not ventilated (closed circles), infected; control group with *S. pneumoniae* pneumonia not ventilated (open diamonds), LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation (closed triangles), HV_T-ZEEP; group with pneumonia and

ventilator-induced lung injury (inverted closed triangles). # $p<0.05$ infected control group and the LV_T-PEEP vs. HV_T-ZEEP.

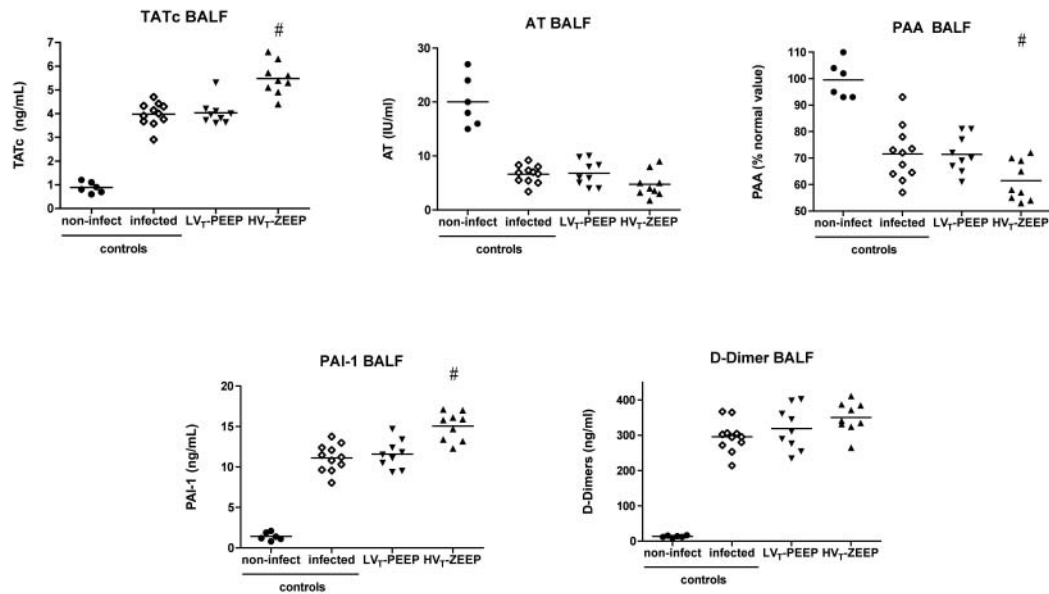


Figure 5

Effect of pneumonia alone and mechanical ventilation and pneumonia on thrombin-anti-thrombin complex (TATc) and plasminogen activator activity (PAA) in serum. Non-infect; control group without pneumonia and not ventilated (closed circles), infected; control group with *S. pneumoniae* pneumonia not ventilated (open diamonds), LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation (closed triangles), HV_T-ZEEP; group with pneumonia and ventilator-induced lung injury (inverted closed triangles). # $p<0.05$ infected control group and the LV_T-PEEP vs. HV_T-ZEEP.

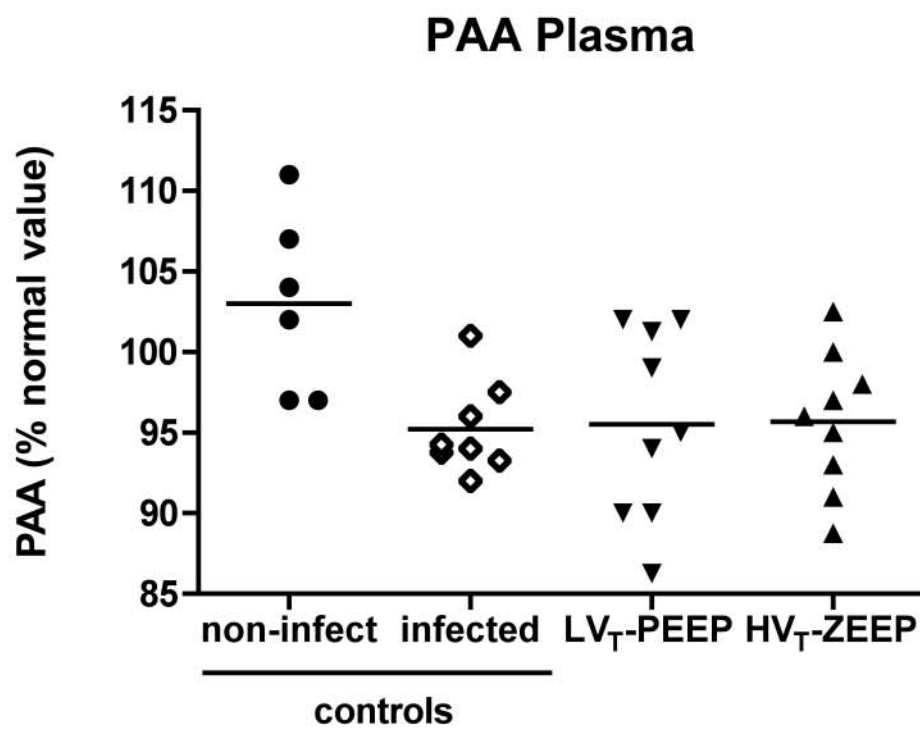
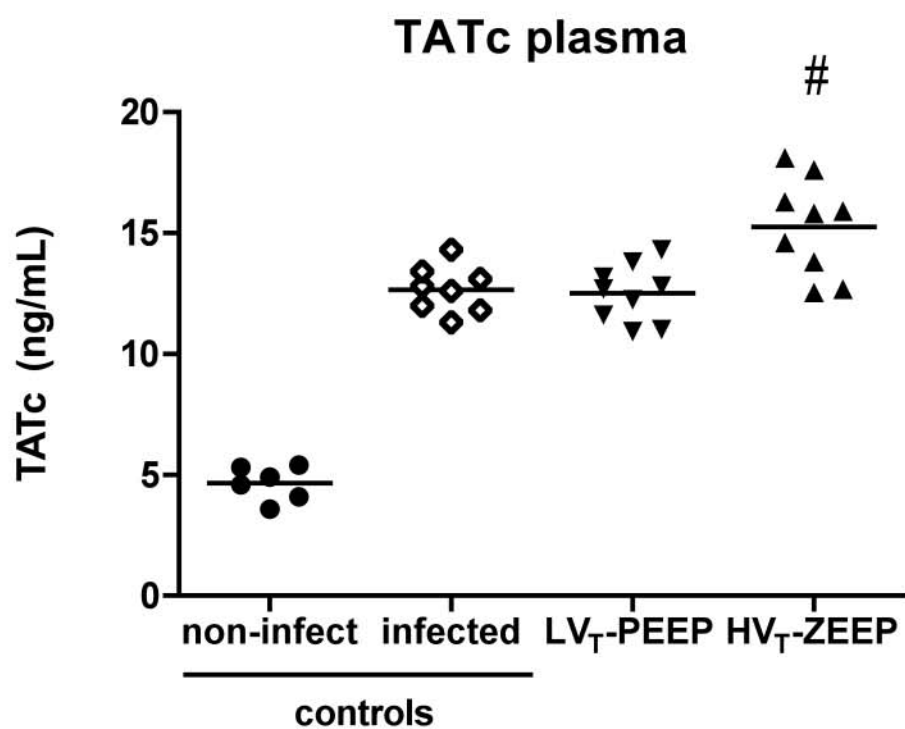


Figure 6

Effect of pneumonia alone and mechanical ventilation and pneumonia on tumor necrosis factor (TNF) and interleukin (IL)-6 in bronchoalveolar lavage fluid and serum. Non-infect; control group without pneumonia and not ventilated (closed circles), infected; control group with *S. pneumoniae* pneumonia not ventilated (open diamonds), LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation (closed triangles), HV_T-ZEEP; group with pneumonia and ventilator-induced lung injury (inverted closed triangles). # $p < 0.05$ infected control group and the LV_T-PEEP vs. HV_T-ZEEP.

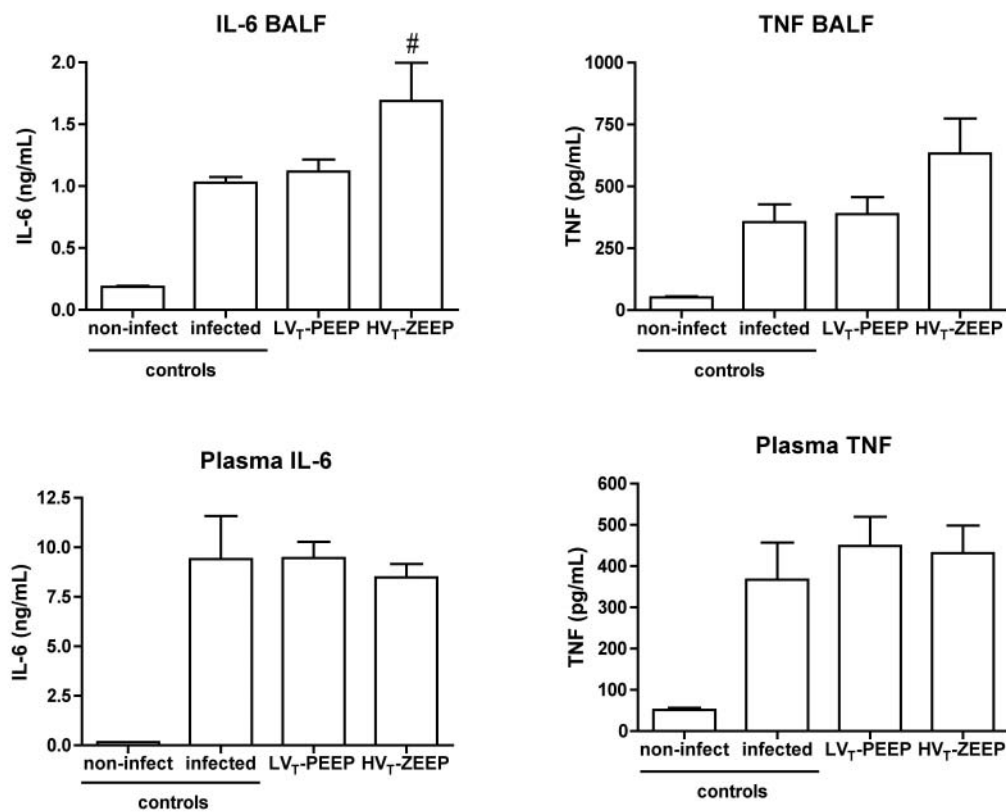


Figure 7

A) Lung injury score data are shown as mean \pm SEM. Infected; control group with *S. pneumoniae* pneumonia not ventilated, LV_T-PEEP; group with pneumonia and ventilated with lung protective mechanical ventilation, HV_T-ZEEP; group with pneumonia and ventilator-induced lung injury. # indicates $p < 0.05$ HV_T-ZEEP group versus LV_T-PEEP group. Representative sections of lungs stained with hematoxylin-eosin: B) Infected, C) LV_T-PEEP and D) HV_T-ZEEP magnification either 50X or 400X as indicated.

