

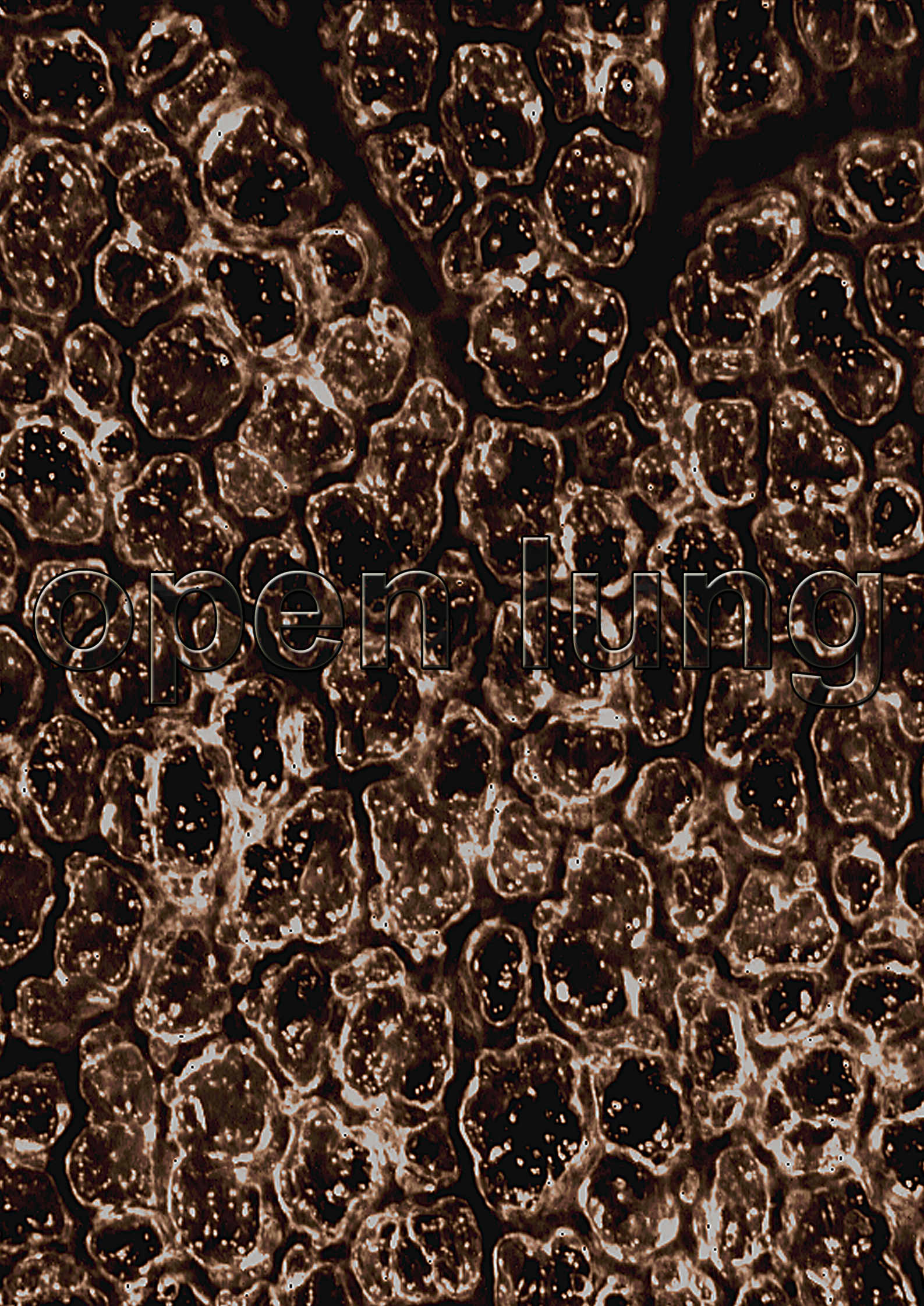
**Diagnostic and Therapeutic Aspects of
Acute Lung Injury:
Experimental Studies**

closed lung

R. A. Lachmann



recruiting...



open lung

Propositions belonging to the PhD thesis:

**Diagnostic and Therapeutic aspects of Acute Lung Injury:
Experimental Studies**

Robert Alexander Lachmann

1. There is no universal best PEEP (this thesis).
2. In terms of bacterial translocation, ventilating a collapsed (atelectatic) lung is as bad as ventilating a lung at high functional residual capacity (this thesis).
3. Treating a bacterial pneumonia solely with exogenous surfactant might not be a good idea after all (this thesis).
4. Treating a bacterial pneumonia using exogenous surfactant as carrier for intratracheal antibiotics seems to be an effective alternative to systemic antimicrobial therapy (this thesis).
5. The negative effects of ventilation-induced lung injury may be selectively blocked by biochemical means (this thesis).
6. Nowadays, everybody can be a creative artist due to modern technology.
7. To ensure social harmony within a hiking group, everybody in the group must walk at his/her own pace.
8. Never try to question or solve problems with software - rather look for workarounds!
9. The introduction of a basic income for all people of a country is the only way to cope with the future technology-dominated job market where machines will do all the “classical” jobs humans used to do.
10. The “*zwembadincident*” cost the Dutch football team the title in 1974.
11. Flying an airplane is easier than driving a car.

**Diagnostic and Therapeutic aspects of Acute Lung Injury:
Experimental Studies**

R.A. Lachmann

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Cover: The front shows a collapsed (atelectatic) lung area (pictures taken using intravital microscopy).

The middle picture shows this lung area during a recruitment maneuver.

The back shows the same lung area after lung recruitment. Alveoli and capillaries are visible.

CD front: The picture on the CD shows a section of the microscopic image on the back cover.

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**Diagnostic and Therapeutic Aspects of Acute Lung Injury:
Experimental Studies**

Diagnostische en therapeutische aspecten van acute longschade:
experimentele studies

Proefschrift

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Für meine Eltern...

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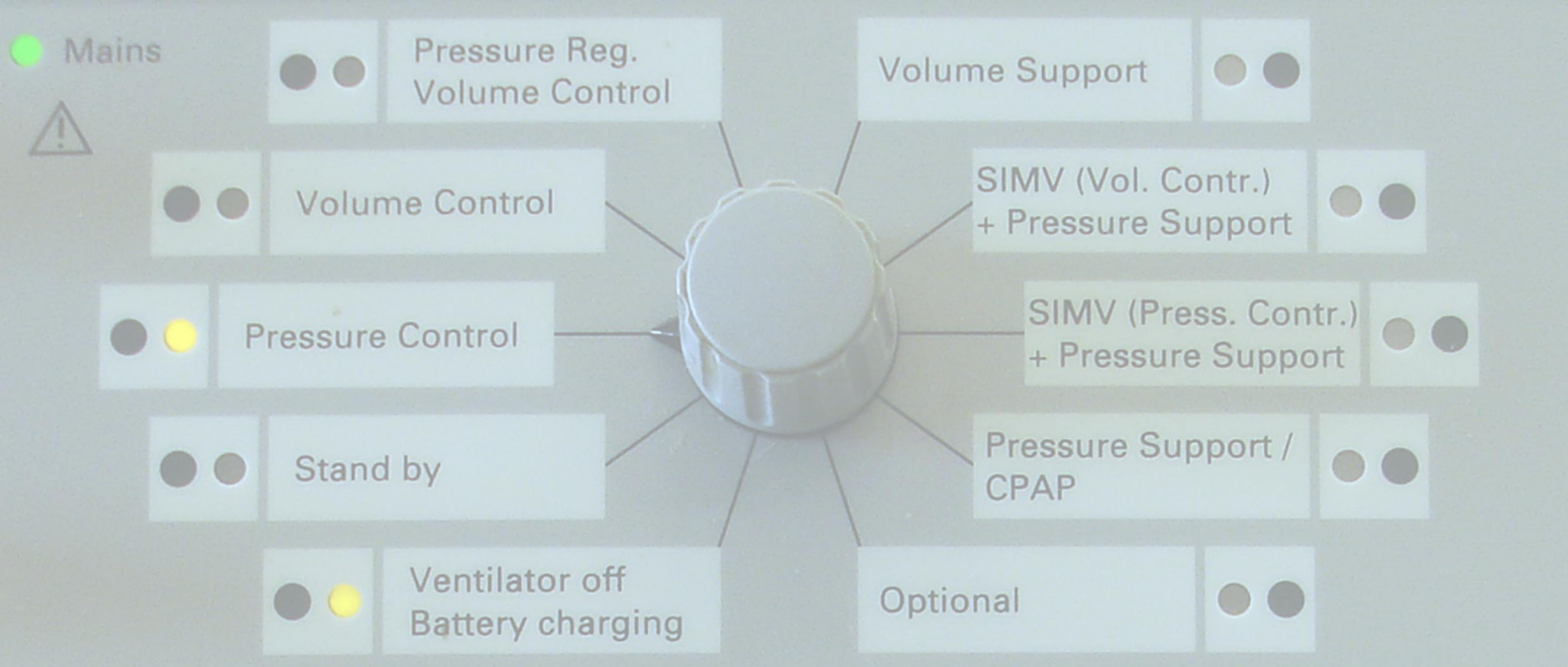
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Preface

Almost 40 years have passed since the acute respiratory distress syndrome (ARDS) was first described in 1967 by Ashbaugh and co-workers. Since then, the pathophysiology of acute lung injury (ALI) and ARDS has been under extensive investigation yielding increasing knowledge about the mechanisms of this potentially fatal disease. It has been demonstrated that pathological changes to the endogenous surfactant system play a major role in the development and progression of ARDS. An impaired surfactant system will also increase the likelihood that mechanical ventilation will be required to ensure that the patient is supplied with sufficient oxygen. However, we have only recently begun to understand the effects that mechanical ventilation can have on the outcome of ARDS. There has been growing awareness concerning the issues of ventilator-induced lung injury leading to the general acceptance of avoiding high tidal volumes with high pressure amplitudes. But the question as to how to optimally ventilate ARDS patients remains, and is still a source of controversy.

With new analyzing techniques, the most recent advances in ARDS research have been made on the sub-cellular level revealing various signal transmission pathways of immune responses, emphasizing the important role of inflammation on the development and/or progression of ARDS. However, the high mortality rates (~30-50%, depending on the source) of ARDS have hardly changed since its first description, in spite of the latest knowledge that has been incorporated into different therapeutical strategies.

This dilemma has only recently been confirmed again in an epidemiologic study (October 2005, Rubenfeld et al.) that investigated the incidence and outcomes of ALI in a region of the USA. That study showed that ALI and ARDS are more common than previously reported, with mortality rates of 38.5% for ALI and 41.1% for ARDS. One may therefore ask why the substantial advances in the pathophysiological knowledge about ALI and ARDS have not yet led to therapeutical strategies that improve outcome. The most probable answer is that ALI and ARDS are very complex and diverse diseases with many different aspects which act on and influence the pathology. Bringing together current and future knowledge will be a key element in fully understanding the extent of ALI and ARDS, which hopefully will increase the chances for successful treatment in the future. This thesis offers an additional piece in the puzzle by focussing on a small selection of aspects involved in the development, progression and possible treatments of ALI and ARDS.



Resp. pattern

Measured freq. b/min
50

Set freq. b/min
50

Insp. period s
0.40

Insp. flow l/s

Chapter 1

Ventilation-induced lung injury and its prevention

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Introduction

Mechanical ventilation has become an important and life-saving therapy in the treatment of premature neonates and patients with an impaired pulmonary function; in particular in patients with the acute respiratory distress syndrome (ARDS), which is characterized by acute respiratory failure and permeability changes of the alveolo-capillary membrane. However, already early after its introduction, it has become realized that certain modes of mechanical ventilation lead to decreases in lung compliance and gas exchange¹, and atelectasis, pulmonary edema, pneumonitis and fibrosis²⁻⁶. Therefore, the realization is growing, that much of the pathophysiological changes seen in ARDS, may be a consequence of our ventilatory interventions in combination with surfactant changes and surfactant changes due to mechanical ventilation, rather than the progression of the underlying disease. Unfortunately, the selection of modes of ventilation still varies considerably⁷.

There is now evidence that new approaches to mechanical ventilation may contribute to improved survival in ARDS^{8, 9} and prognostic factors on ARDS survival are related to pulmonary gas exchange^{10, 11}. This paper focuses on the interaction of surfactant abnormalities with mechanical ventilation.

Ventilator-Induced Lung Injury (VILI)

Surfactant changes due to mechanical ventilation

Pioneering work of Mead in 1959 showed that mechanically ventilated dogs had a progressive fall in pulmonary compliance¹²; such mechanical changes were related to the pulmonary surfactant system as shown by Greenfield and co-workers who demonstrated increased surface tensions of lung extracts in dogs ventilated at peak inspiratory pressures of 28-32 cmH₂O for 1 to 2 hours¹³. A subsequent report by Sladen et al. showed that also patients ventilated for long periods suffered from an increased alveolar-arterial oxygen gradient, and a fall in respiratory system compliance¹⁴. Early studies by Benzer have demonstrated that rabbits ventilated with an open thorax at a peak inspiratory pressure of 30 cmH₂O with 5 cmH₂O of PEEP or with a closed thorax without PEEP had a better preserved surfactant system at the end of a 75-minute ventilation period than animals ventilated with an open thorax without PEEP¹⁵; these observations were extended in a subsequent report by Woo and Hedley-Whyte who observed pulmonary edema foam in the airways of open-chest dogs ventilated with large tidal volumes, whereas the same ventilator settings in closed-chest animals induced no such abnormalities¹⁶. Two primary mechanisms of surfactant inactivation by mechanical ventilation have been described. First, mechanical ventilation was shown to enhance surfactant release from the

pneumocyte type II into the alveolus [17-20](#). This material is subsequently lost into the small airways as a result of compression of the surfactant film when the surface of the alveolus becomes smaller than the surface occupied by the surfactant molecules [21, 22](#) (Figure 1). A second mechanism to describe the surfactant changes associated with mechanical ventilation is based on the observation that the alveolar surface area changes associated with mechanical ventilation, result in the conversion of surface active large surfactant aggregates into non-surface active small surfactant aggregates [23-27](#) (Figure 2). Surfactant changes due to mechanical ventilation are reversible as a result of a metabolically active process, involving de novo production of surfactant [18](#). It probably involves a balance between secretion and production of large aggregates, and uptake clearance and reconversion of small aggregates in the pneumocyte type II [28](#).

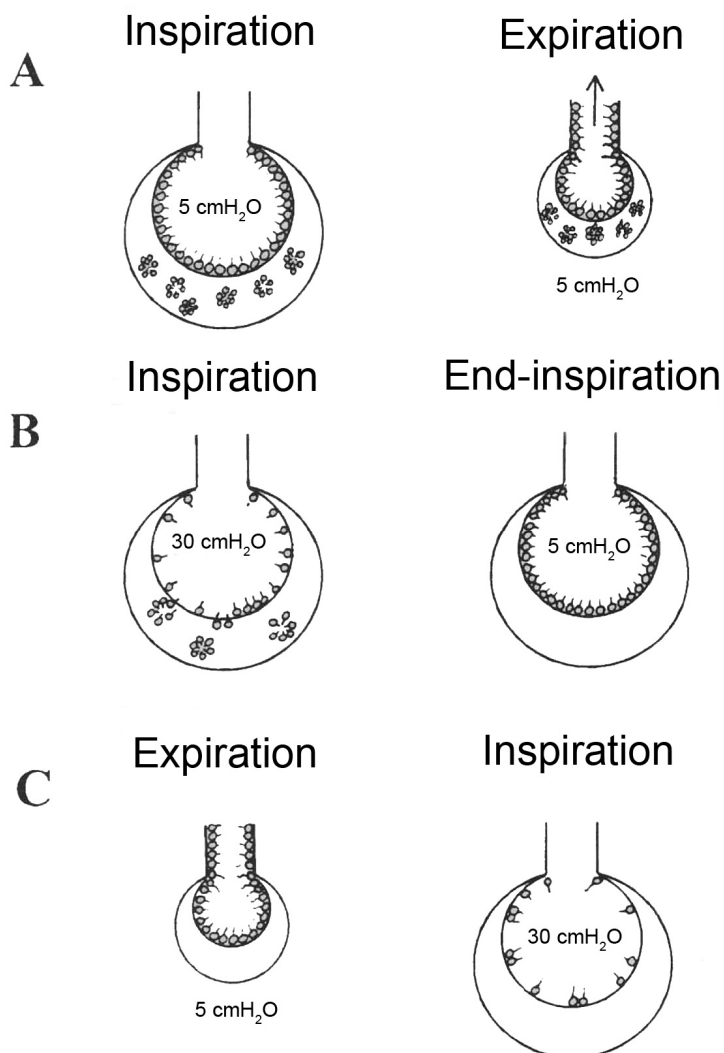


Figure 1.

(A) Balance between synthesis, release and consumption of surfactant in the healthy lung. The pressure values given represent the intrapulmonary pressure needed to open up the alveolus. At the surface and the hypophase (micelles), there are sufficient molecules of surfactant. These micelles deliver the surfactant necessary to replace the molecules squeezed out during expiration.

(B) Imbalance between synthesis, release and consumption of surfactant due to artificial ventilation. At the beginning of inspiration, there is an apparent deficiency of surfactant molecules but there is a respreading of molecules stored in the hypophase of the surfactant layer. At the end of inspiration there is, in principle, enough surfactant on the surface.

(C) With the next expiration, surface active molecules are squeezed out and no surface active molecules are left in the hypophase for respreading, creating the situation where a serious surfactant deficiency follows.

Consequences of surfactant changes for fluid balance and solute permeability of the alveolo-capillary barrier

The hypothesis proposed by Pattle and Clements ^{29, 30} that loss of active surfactant with an increase in alveolar surface tension results in a decrease in pericapillary pressure was proven by demonstrating increased transmural filtration pressure, in experiments in which surfactant impairment was induced by cooling the lung and ventilation with large tidal volume ³¹ or by aerosolation of detergent ³². However, surfactant dysfunction has also been shown to increase the permeability of the alveolo-capillary barrier to small solutes e.g. technetium-99m-labelled diethylene triamine pentaacetic acid (^{99m}Tc-DTPA) in the absence of other substantial changes in the function of the alveolocapillary unit ³³, and increased surfactant content in healthy ventilated rabbits was shown to reduce the permeability for the same molecule ³⁴. Surfactant is not only rate-limiting for the transfer of small solutes; studies in both premature animals ^{35, 36} and adult surfactant-depleted animals ³⁷ have shown that surfactant treatment before starting mechanical ventilation substantially reduces the transfer of albumin over the alveolo-capillary barrier. Studies in smokers compared to healthy controls have shown that a reduction in the

Non-active/active total phosphorus ratio

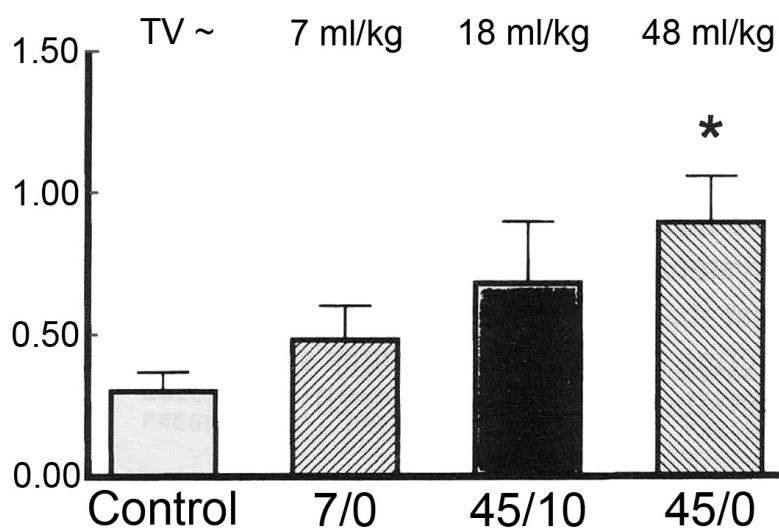


Figure 2. Ratio of non-active to active total phosphorus (P) in rats ventilated for 20 minutes with peak inspiratory pressures/positive end-expiratory pressures of: 45/0; 45/10; 7/0 and non-ventilated controls. Group 45/0 had a significant conversion of active to non-active total P during the ventilation period compared with controls (from reference ²⁵).

amount of phosphatidyl choline in broncho-alveolar lavage of smokers is associated with changes in the permeability of the alveolo-capillary barrier to ^{99m}Tc-DTPA ³⁸. These studies indicate that surfactant has a primary role in the regulation of the permeability of the alveolo-capillary barrier to small solutes and protein. This may be due to both a direct action of surfactant on the alveolo-capillary barrier and a reduction of the structural damage caused by mechanical ventilation due to the presence of surfactant.

Disturbed fluid balance over the capillary barrier due to mechanical ventilation

Functional integrity of both the endothelium and epithelium is a prerequisite for maintaining a normal fluid balance at the alveolo-capillary membrane. Both increased capillary filtration pressure and altered microvascular protein permeability have been shown to contribute to pulmonary edema after lung overinflation. Studies in open-chest large animals, which indirectly calculated the capillary filtration pressure from measurements of mean pulmonary artery and left atrial pressures after lung overinflation at peak inspiratory pressures of around 60 cmH₂O, demonstrated a mild increase in mean transmural microvascular pressure as a result of overinflation when compared to normal conditions [39](#), [40](#). However, any increase in transmural microvascular pressure will have a dramatic effect on edema formation when the microvascular barrier has altered sieving properties [41](#). The three basic forces acting on the capillary wall which can eventually result in loss of its functional integrity have been reviewed by West [42](#).

1) An increase in the circumferential tension (which is directly proportional to the transcapillary wall pressure and the capillary radius and inversely proportional to the wall thickness). It may be speculated that ventilation-induced surfactant impairment with alveolar collapse, hypoxic vasoconstriction and redistribution of blood flow to selected capillaries, may increase transcapillary wall pressure and thus circumferential tension in those capillaries. In principal this will result in hydrostatic edema, but it may be speculated that it becomes of the permeability type if transcapillary pressure reaches 40 mmHg or above. Injury at such pressures is not limited to the endothelial cells but also causes breaks in type I cells [43](#).

2) Surfactant inactivation due to mechanical ventilation results in loss of the supportive 'hoop' function by surfactant on the capillary wall. An increase in surface tension therefore causes a reduction in perivascular pressure, with an increase in distending pressure on the capillary. Nieman et al. showed that the number of alveoli with continuous blood flow (zone III conditions) increases after lung lavage with a detergent solution elevating surface tension [44](#).

3) Longitudinal tension on the capillary due to lung overinflation. Fu and colleagues have shown that increasing lung volume by increasing transpulmonary pressure from 5 to 20 cmH₂O at a constant capillary pressure of 32 cmH₂O resulted in a significant increase in the number of endothelial and epithelial type I breaks [45](#). The increase in the number of endothelial breaks produced by equivalent increases in transpulmonary pressure and capillary transmural pressure were similar. Thus, vascular pressures too low to affect microvascular permeability at low lung volume may increase microvascular permeability when the lung volume is sufficiently increased.

Surfactant changes affect endothelial susceptibility to breaks by mechanical ventilation

Many studies in open and closed chest animals using different approaches have shown that lung overinflation is associated with changes in microvascular permeability [39](#), [40](#), [46-49](#). The existence of a pressure threshold above which microvascular permeability changes occur has been suggested [50](#), although others suggested the absence of a well-delimited pressure or volume threshold [51](#). Independent of this, it has become clear that microvascular injury secondary to ventilation occurs at much lower airway pressures and volumes in isolated perfused lungs with inactivated surfactant due to dioctyl succinate, as compared to ventilation of healthy lungs [52](#). These studies suggest that lungs with an impaired surfactant system are more susceptible to overinflation than healthy lungs and that minor surfactant alterations, such as those produced by spontaneous ventilation during prolonged anesthesia [53](#), [54](#) are sufficient to synergistically increase the harmful effects of overinflation on permeability of the endothelial barrier [55](#). Similarly, whereas either oleic acid or mechanical ventilation in isolated lungs did not significantly affect capillary permeability, the combination of the two did [56](#). Studies in intact animals suggested a synergism rather than additivity between lung injury induced by mechanical ventilation and α -naphthyl-thiourea [57](#) in inducing endothelial permeability.

Disturbed fluid balance over the epithelial barrier due to mechanical ventilation

It is known that more than the endothelium or interstitial spaces, the epithelium is rate-limiting for solute and fluid movement between blood and alveolus [57](#), [58](#). Effects of overinflation on epithelial permeability have been studied in fluid-filled in situ lobes, to exclude the effect of surface tension. As the epithelium is progressively stretched during static inflation there is a non-reversible opening of water-filled channels between alveolar cells resulting in free diffusion of small solutes and even albumin across the epithelial barrier [59-61](#). Such changes were shown to occur only at high distending pressures and have been attributed to peak inspiratory epithelial overstretching which occurs due to inflation in the supra-physiological range only [61](#). Experimental studies with small solutes like ^{99m}Tc -DTPA have shown that the rate of clearance of this tracer from the alveolar space increases with increases in lung volume, whether caused by large tidal volume ventilation [62](#) or PEEP [63](#). Due to damage of both the epithelial and endothelial barrier, surfactant components may be lost into the bloodstream [64-66](#). More importantly, protein will accumulate intra-alveolarly which results in dose-dependent inhibition of surfactant [67-70](#). As surfactant is rate-limiting for the transfer of proteins over the alveolo-capillary barrier, loss of surfactant function will lead to further protein infiltration. This may result in a self-triggering mechanism of surfactant inactivation.

Surfactant changes as an explanation for epithelial breaks due to mechanical ventilation: repeated alveolar collapse and reexpansion

As discussed above, endothelial and epithelial overstretching with widening of intracellular junctions is considered the basic mechanism for loss of alveolo-capillary barrier function. However, one idea of ventilation-induced lung injury and epithelial stretching goes back to the pioneering work of Mead who demonstrated that, due to pulmonary interdependence of alveoli (Figure 3), the forces acting on the fragile lung tissue in non-uniformly expanded lungs are not only the applied transpulmonary pressures, but rather the shear forces that are present in the interstitium between open and closed alveoli ⁷¹. An alveolus with surfactant impairment would be predisposed to end-expiratory alveolar collapse and prone to be affected by such 'shear forces'. Shear forces, rather than end-inspiratory overstretching, may well be the major reason for epithelial disruption and loss of barrier function of the alveolar epithelium and considerable increases in regional microvascular transmural pressure. Important evidence for this mechanism comes from the findings that ventilation at low lung volumes can also augment lung injury in lungs with an impaired surfactant system ⁷² a concept first proposed by Robertson et al. ⁷³. A recent study in a model of subtle surfactant perturbation by dioctyl sodium sulfosuccinate showed that surfactant changes make the lung vulnerable to lung parenchymal injury by mechanical ventilation ⁷⁴. These studies confirm earlier work of Nilsson et al. in ventilated premature newborn rabbits with a primary surfactant deficiency. Foetuses treated with surfactant before receiving mechanical ventilation had less bronchiolar epithelial lesions in comparison to non-surfactant treated controls ⁷⁵.

Positive end-expiratory pressure (PEEP)

Effect of PEEP on ventilation-induced lung injury

Initial studies have investigated the effect of increasing levels of PEEP at constant tidal volume ventilation, which resulted in higher end-inspiratory pressures and volumes. Such studies found that increasing levels of PEEP reduced shunt ^{76, 77} and improved oxygenation and lung mechanics ⁷⁸ which was attributed to reopening of flooded alveoli with redistribution of edema fluid from flooded alveoli into the interstitial spaces ⁷⁹⁻⁸¹. Such studies, however, also demonstrated that the use of high PEEP levels did not reduce ^{76, 78, 82} or even increase edema formation ^{77, 83}. These findings have been reported in both isolated perfused lungs ⁷⁶ and in closed-chest healthy animals ⁷⁸ and closed-chest animals with different forms of lung injury induced by bronchial hydrochloric acid administration ⁷⁷, alloxan ⁸², oleic acid ⁸⁴ or hydrostatic edema due to lobar venous occlusion ⁸³. Overinflation due to PEEP is probably the explanation

for the lack of reduction or even worsening of edema reported with PEEP during such experiments ⁸⁵. However, it has now been unequivocally demonstrated in different animal models that ventilation with PEEP at lower tidal volumes results in less edema than ventilation without PEEP and a higher tidal volume for the same peak or mean airway pressure ^{6, 47, 85, 86} and that, more specifically, PEEP prevents alveolar flooding ^{25, 47}. Studies by Dreyfuss et al. in rats ventilated at peak inspiratory pressure of 45 cmH₂O have shown that damage due to mechanical ventilation begins at the endothelial side after 5 min and rapidly progresses to the epithelium after 20 min ⁴⁹. A subsequent study showed a reduction of endothelial injury and the preservation of the structure of the alveolar epithelium by use of 10 cmH₂O of PEEP, which was accompanied by a lack of alveolar flooding ⁴⁸.

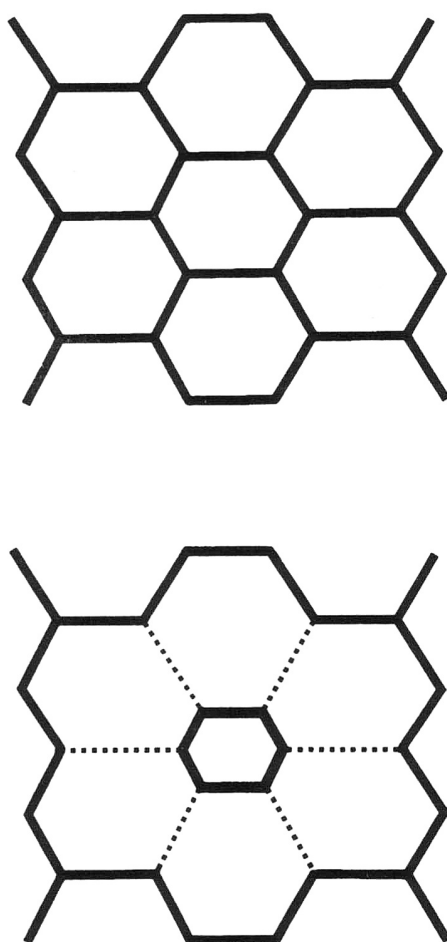


Figure 3.

Shear forces are caused between open and closed alveoli due to pulmonary interdependence of alveoli. This figure shows the difference between mechanical ventilation of normal alveoli (upper panel) and mechanical ventilation of the same alveolar unit after surfactant inactivation (lower panel), which results in end-expiratory collapse (*adapted from reference 71*).

Reduced microvascular filtration due to capillary compression by PEEP

Several experiments in closed-chest animals have suggested that PEEP reduces microvascular filtration pressure due to a decrease in cardiac output ^{76, 82, 87}. It was shown in rats ventilated at peak inspiratory pressure of 45 cmH₂O that the main determinant of lung edema formation is the end-inspiratory lung volume independent of the level of PEEP ⁸⁸. Infusion of dopamine to correct the drop in systemic arterial pressure that occurs with PEEP was shown to partially abolish the reduction in pulmonary edema

by PEEP ⁸⁸. The effect of PEEP in reducing protein infiltration and permeability of the alveolo-capillary barrier was attributed to a decrease in lung capillary hydrostatic pressure and, therefore, filtration pressure ⁸⁸. Such a mechanism occurs at supraphysiological PEEP levels, higher than the level necessary to compensate for the retractive forces of the alveolus, and is attributable to compression of the capillary by adjacent alveoli. However, pulmonary artery pressure or cardiac output were not recorded in that study ⁸⁸ and loss of the endothelial and

epithelial barrier function was not differentiated. Moreover, despite similar arterial pressures, the animals ventilated with PEEP that received dopamine had less edema than animals ventilated without PEEP, suggesting that reduced filtration by capillary compression is not the only reason for the reduction in edema by PEEP and that other mechanisms are acting.

PEEP and the surfactant system: Reduced filtration due to surfactant preservation

Experiments in the same rat model of overinflation have shown a significant conversion of active into non-active surfactant aggregates compared to non-ventilated controls after lung overinflation; 10 cmH₂O PEEP was shown to prevent a significant conversion of large aggregates into small aggregates compared with non-ventilated controls ²⁵. This latter study suggests that the beneficial effect of PEEP in reducing protein infiltration after overinflation at peak inspiratory pressure of 45 cmH₂O without PEEP in rats is partially attributable to a reduced filtration by surfactant preservation ²⁵. Two basic mechanisms have been described in literature which explain the surfactant preserving effect of PEEP during mechanical ventilation. Studies by Wyszogrodski et al. have shown that PEEP prevents a decrease in lung compliance and surface activity of lung extracts, indicating a prevention of loss of alveolar surfactant function during lung overinflation ²¹. It was suggested that PEEP prevents alveolar collapse and thus keeps the end-expiratory volume of alveoli at a higher level, thereby preventing excessive loss of surfactant in the small airways by a squeeze-out mechanism during expiration (Figure 1) ^{89,90}. Successive studies by Veldhuizen and colleagues have shown that the rate of conversion of surfactant large into small aggregates is dependent on tidal volume and on time ⁹¹; changing the respiratory rate ⁹¹ or the level of PEEP ²⁴ did not affect surfactant conversion. These studies suggest that the preservation of the surfactant system by PEEP comes from the reduction in cyclic changes in surface area by PEEP (Figure 2). It should be noted, however, that at a higher functional residual capacity (FRC), comparable changes in tidal volume are accompanied by smaller surface area changes compared to the same volume changes at lower FRC. To further test the hypothesis that reduced filtration due to surfactant preservation is responsible for the reduction of edema by PEEP, our group conducted a study in which high peak inspiratory pressure ventilation without PEEP was preceded by administration of high amounts of exogenous surfactant ⁹². It was shown that an amount of 200 mg/kg bodyweight surfactant preserved oxygenation and lung mechanics after 20 minutes of overinflation at peak inspiratory pressures of 45 cmH₂O without PEEP. Although 400 mg/kg bodyweight surfactant did not reduce the lung tissue content of Evans blue dye it was shown to reduce intra-alveolar accumulation of Evans blue dye ⁹² (Figure 4). Evans blue dye extravasation has previously been

shown to have a good correlation with extravasation of ^{125}I -Albumin ⁹³. These data provide strong evidence that, besides peak inspiratory overstretching after lung overinflation, surfactant inactivation plays a key role in ventilation-induced intra-alveolar edema formation and that the effect of PEEP in reducing lung permeability to protein is at least partially attributable to its effect on preservation of the surfactant system. In a subsequent study we were able to demonstrate that exogenous surfactant can be used to treat ventilation induced lung injury once established ⁹⁴.

Splinting open alveolar lung units with an increased collapse tendency by PEEP

The utilization of PEEP to splint open the airways and alveoli at end-expiration in surfactant-deficient lungs may markedly reduce lung injury. Studies in both saline-lavaged isolated perfused rat lungs ⁷² and saline-lavaged intact animals ^{95, 96} have shown that ventilation strategies which keep the alveoli open throughout the respiratory cycle by sufficiently high levels of PEEP induce significantly less morphological injury with better preservation of pulmonary compliance than strategies in which alveolar collapse is allowed at end-expiration.

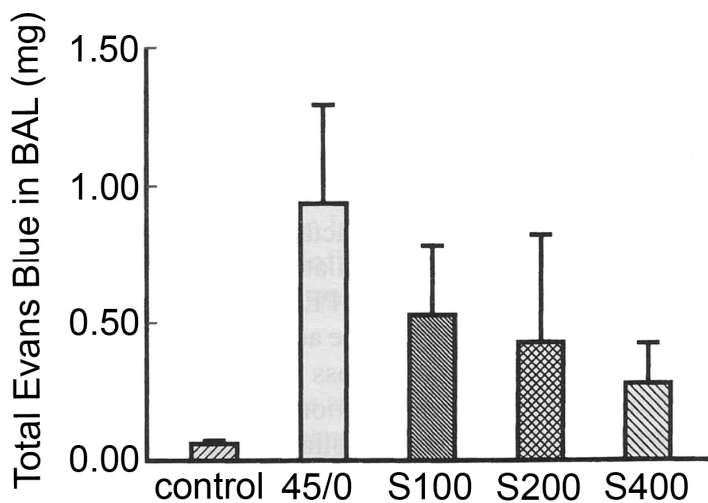


Figure 4. This figure shows Evans blue dye recovery after 20 min from the broncho-alveolar lavage fluid after intravenous injection of 30 mg/kg Evans blue in non-ventilated control rats (control), and rats ventilated with Peak pressure/PEEP of 45/0 which received 100 mg/kg (S100), 200 mg/kg (S200), 400 mg/kg (S400) or no intratracheal surfactant (45/0) prior to mechanical ventilation.

Although healthy lungs do not seem to be damaged when terminal units are repeatedly opened or closed for short periods by negative end-expiratory pressure (which nevertheless reduces compliance and alters gas exchange ⁹⁷), it becomes clear from what is discussed above, that early surfactant changes, which may be induced by mechanical ventilation itself, predispose lungs for ventilation-induced lung injury by repeated opening and closure of alveolar units ⁷⁴.

Ventilation-induced lung injury and its relationship to systemic disease

The mortality rate from ARDS has not decreased since it was first described ⁹⁸ and mortality rates range from 10% to as high as 90%, with an average of 50% ⁹⁹. Multiple organ failure (MOF) is the leading cause of death in ARDS ¹⁰⁰ and the majority of patients with MOF develops ARDS ¹⁰⁰ as the initial organ failure ¹⁰¹. Therefore, ARDS is now being regarded as an important, causative part of an inflammation-induced systemic disease state that can evolve to MOF, rather than as a sole pulmonary disease process ¹⁰². It is becoming increasingly realized that systemic release of inflammatory mediators ¹⁰³ and bacterial translocation from the gut into the circulation ¹⁰⁴ play a role in MOF. Kolobow et al. suggested that mechanical ventilation may induce local inflammatory reactions in the lung and may possibly, via spread of inflammatory mediators and bacteria, contribute to MOF ¹⁰⁵.

Possible sites of inflammatory mediator release

Experiments with granulocyte-depleted animals have shown that a significant amount of ventilation-induced lung injury may be mediated by activated granulocytes ¹⁰⁶ and those ventilation strategies that use high volume ¹⁰⁷ and/or cyclic collapse and reexpansion of alveolar units ^{108, 109} increase neutrophil influx and activation. Decreasing neutrophil infiltration by glucocorticoids or inhibition of neutrophil adhesion by leumedins reduces ventilation-induced lung injury ^{110, 111}. The concept of ventilator-induced mediator expression as a result of either damage to the endothelial or epithelial cells or stimulus of stretch receptors present on endothelial cells ¹¹², macrophages ¹¹³ and epithelial cells ¹¹⁴ is new (the exact signal transduction pathways have been reviewed elsewhere ⁶).

Ventilation-induced mediator release

There is now compelling evidence for the release of cytokines during mechanical ventilation. High frequency oscillation (HFO) in surfactant-depleted rabbits, which reduces shear stress, has been shown to induce lower levels of the inflammatory chemical mediators platelet-activating factor (PAF), thromboxane (TX) B₂ and 6-keto-prostaglandin (PG) F_{1β} in the lavage fluid compared to conventional mechanical ventilation (CMV) ¹¹⁵. Interestingly, this could not be demonstrated in healthy unlavaged lungs ¹¹⁵ with an intact surfactant system. Others could not show a difference between thromboxane A₂ and prostacyclin in the perfusate of oleic acid-injured isolated rabbit lungs ventilated with high and low tidal volume (Vt) ¹¹⁶. Similarly, an increase in TNF-α mRNA in the intra-alveolar cells of surfactant-depleted rabbits after 1 h of CMV was absent when such rabbits were ventilated with HFO ¹¹⁵. The importance of such

findings was demonstrated by the fact that instillation of anti tumor necrosis factor alpha could attenuate ventilator-induced lung injury [117](#). A study by Tremblay and co-workers in isolated lungs investigated the effect of different ventilation strategies on lung inflammatory mediator expression and production of cytokines TNF- α , IL-1 β , IL-6, IL-10, MIP-2 and γ -IFN in the presence and absence of a preexisting inflammatory stimulus [118](#). It was shown that the use of high peak inspiratory lung volumes and not using PEEP during mechanical ventilation have a synergistic effect on the release of pro-inflammatory mediators from the lung tissue into the airways. Ten cmH₂O of PEEP at comparable peak inspiratory lung volumes or lowering peak inspiratory lung volume when ventilating with zero PEEP reduced these cytokine levels [118](#). The same authors also found cytokine expression and release as a result of mechanical ventilation with different ventilator strategies [119, 120](#). However, in an in vivo rat study our group was not able to demonstrate TNF- α expression as a result of mechanical ventilation with different ventilation strategies [121](#). Only after stimulation with lipopolysaccharide animals became positive for TNF- α [121](#). Similar results were found by Richard et al. [122](#). Studies by von Bethmann et al. have shown that prostacyclin, tumor necrosis factor α and interleukin-6 are released into the lung perfusate of isolated perfused and ventilated mouse lungs after artificial ventilation [123](#). For TNF release a continuous stimulation by hyperventilation is necessary whereas a short period of 30 minutes of hyperinflation is sufficient to stimulate release of IL-6 for the next 120 minutes [124](#). More and more studies are appearing, which suggest a role for surfactant changes in the cascade of ventilation-induced lung injury and the release of inflammatory mediators [125-127](#). From such studies it may be hypothesized that mechanical ventilation serves to initiate and/or propagate an inflammatory response in the lung that acts as a nidus for the development of a systemic inflammatory response and that MOF could, to a certain degree, therefore be caused by non-optimal mechanical ventilation.

Animal investigations by Narimanbekov et al. suggest that treatment with antibodies directed against IL-1 reduced albumin infiltration, elastase expression and neutrophil counts in rabbits subjected to pulmonary lavage and 8 hours of hyperoxia and hyperventilation [128](#). However, no effect was seen on deterioration of lung compliance and oxygenation which suggests that the different effects of ventilation-induced lung injury may be directed by different molecules.

Ranieri et al. showed that mechanical ventilation designed to minimize ventilator-induced lung injury using high PEEP levels and low end-inspiratory stretch could markedly attenuate the cytokine response in ARDS patients compared to conventional ventilation strategy [129](#). In contrast to ARDS, inflammatory mediator release as a result of different forms of mechanical ventilation could not be shown in patients with normal pulmonary function [130](#). Although it may

be argued that these authors did not measure cytokine levels of broncho-alveolar lavage fluids and the time period of 1 hour may have been too short to elicit a difference in cytokine levels, the authors concluded that previous lung damage seems to be mandatory to cause an increase in plasma cytokine levels after 1 hour of high tidal volume mechanical ventilation.

Numerous biological markers have been identified which may be used to evaluate damage to the alveolo-capillary barrier [131](#). For the endothelium, many specific markers are available [131](#), but at present only increased protein permeability of radio-active tracers from the alveolar lung compartment into the circulation evaluates the epithelial integrity, which is rate limiting for the transfer of solutes over the alveolo-capillary barrier [131](#). A study by Verbrugge et al. suggests the use of purines as a specific marker of epithelial injury [132](#). The search should continue for highly specific biological markers in the bloodstream, such as are available for other organs, to monitor the metabolic and biological stress inflicted on the lung by our supportive ventilation therapy.

Ventilation-induced bacterial translocation

Based on the observation that mechanically ventilated ARDS patients often develop pneumonia [133](#) and septicemia the question may be raised whether damaging mechanical ventilation can promote bacteremia and/or sepsis. It is conceivable that bacteria more readily gain access to the circulation from damaged lung parenchyma than from previously normal lung tissue [134](#), [135](#). It has been established that preserving end-expiratory lung volume with PEEP has a beneficial effect on the course of infection in terms of reducing bacterial counts recovered from the lung tissue after prolonged mechanical ventilation of lungs inoculated with bacteria [136](#). Moreover, avoiding high peak transpulmonary pressures and preserving end-expiratory lung volume with PEEP has been shown to reduce translocation of *Pseudomonas aeruginosa* [136](#), *Escherichia Coli* [137](#) and *Klebsiella pneumoniae* [138](#) from the lung into the bloodstream (Figure 5). The same principle applies to endotoxin derived from the lung, which may translocate as a result of detrimental forms of mechanical ventilation [139](#).

These data suggest that ventilation-induced changes in the barrier function of the lung epithelium and/or endothelium to bacteria may, to a certain extent, contribute to the development of bacteremia and endotoxemia as it is seen in MOF. Translocation may be due to both an increased translocation of bacteria from the alveolar space directly into the bloodstream or bacterial clearance from the interstitial spaces due to increased lymph flow which promotes drainage of bacteria from the lymphatic's into the bloodstream.

So far, the effects of surfactant therapy on ventilation-induced inflammatory mediator expression and bacterial translocation have not been investigated. Such questions pose a major

challenge for future investigations.

Modes of mechanical ventilation which prevent ventilation-induced lung injury (VILI)

Since its introduction into clinical use more than 40 years ago, mechanical ventilation has proven to be a life-saving method of therapy in intensive care. Yet, it remained a topic of much discussion and controversy because mechanical ventilation involves a disturbance of normal respiratory and cardiovascular function. The experimental data presented above demonstrate that ventilation settings that prevent lung injury in both healthy and diseased lungs should prevent alveolar overdistension and recruit all alveoli and prevent their collapse at end-expiration.

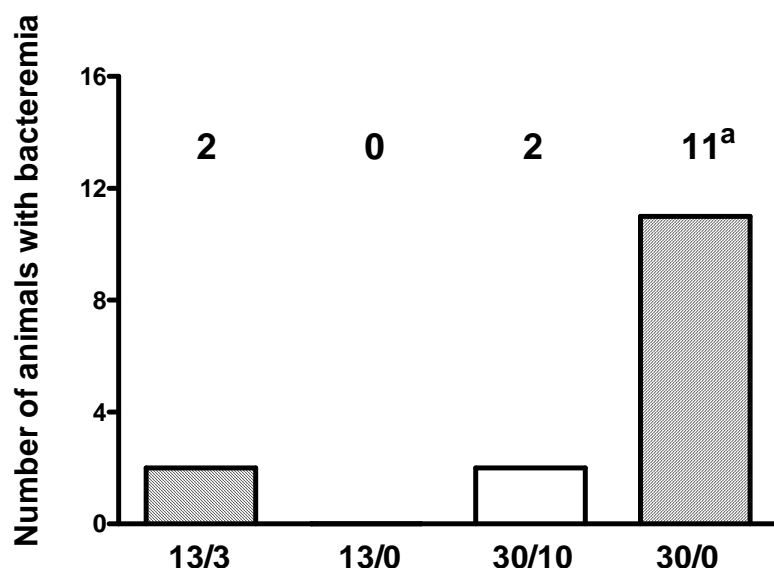


Figure 5.

Number of animals with positive blood cultures for *K. pneumoniae* in 2 mL of blood after three hours of mechanical ventilation of the following peak inspiratory pressures/PEEP settings: 13/3; 13/0; 30/10; 30/0. The animals were inoculated with *K. pneumoniae* 22 hours prior to mechanical ventilation. Group 30/0 had significantly more animals with positive blood cultures than the other groups (from reference [138](#)).

Preventing overdistension

Gattinoni et al. showed that patients with early ARDS and collapsed dependent lung regions, have a reduced volume of aerated lung [140](#). Volume controlled mechanical ventilation will predominantly ventilate this aerated healthy portion of the lung with overdistension in such regions. If one assumes that 75% of the lung is consolidated and only 25% is ventilated, then even small tidal volume ventilation with e.g. 7 mL/kg bodyweight, would result in tidal volumes of 28 mL/kg in such lung regions with a danger of overdistension and further lung impairment. Use of pressure-controlled time-cycled modes of mechanical ventilation in which

the alveolar pressure can never exceed the peak inspiratory pressure set on the ventilator is then preferable to reduce dangerous alveolar overdistension in these lung areas [141](#).

It was recently suggested that to prevent overdistension in ARDS patients, tidal volumes have to be decreased [142](#) and that tidal volume reduction would increase oxygen delivery due to better hemodynamics [143, 144](#).

Ventilation with reduced tidal volumes by end-inspiratory airway pressure limitation in patients with or at risk of ARDS, however, showed no reduction in mortality rate [145, 146](#) except in one study [147](#). However, the reduced mortality in the latter study was probably due to the creation of intrinsic PEEP [148](#). Such findings may be explained by a certain degree of VILI even with small tidal volume ventilation, due to repeated alveolar collapse and reexpansion as discussed below. Hypercapnia may to a certain extent also be responsible for this, because hypercapnia, which has several negative effects [149](#), was accepted as a consequence of tidal volume reduction (except in the ARDS Net study).

The Open Lung Concept

The imperative: “Open up the lung and keep the lung open” from Lachmann’s editorial [150](#) has been quoted many times. The implied rationale, however, has been a matter of debate: Why should we ‘open the lung’? What is ‘an open lung’? In addition, questions concerning the methodology were asked: How can we ‘open the lung’ without risking barotrauma? How should we keep the lung open with the least possible side effects?

What is an open lung?

The open lung is characterized by an optimal gas exchange [150](#). The intrapulmonary shunt is ideally less than 10%, which corresponds to a PaO₂ of more than 450 mmHg on pure oxygen [151-153](#). At the same time, airway pressures are at the minimum that ensure the required gas exchange. Hemodynamic side effects are thus minimized [2, 150](#).

Why should we open the lung?

The classical paper about the acute respiratory distress syndrome by Ashbaugh and colleagues [98](#) describes the consequences of closed lung units: Hypoxemia, intrapulmonary shunt and atelectasis, with high risk of infection, multiorgan dysfunction and finally death [11, 154](#). Ashbaugh encouraged clinicians to reexpand collapsed lung units by high tidal volumes and high levels of PEEP [98](#). However, as already mentioned above, it has been known for many

years that mechanical ventilation itself can also damage the lung [6](#), [155](#), [156](#). The application of high inspiratory pressures and volumes with overdistension of open alveoli for a long time is associated with an increased risk for barotrauma [6](#), [46](#), [47](#), [88](#), [154](#). On the other hand, low levels of PEEP may contribute to ventilation-induced lung injury by allowing alveoli to collapse and reopen during each respiratory cycle [6](#), [46](#), [47](#), [72](#), [88](#), [154](#), [155](#), [157](#), [158](#) and, as explained above, shear forces are the result [71](#), [74](#), [159](#), [160](#). If these findings were true, what then is a safe strategy to open atelectatic lungs?

Physiologic Background

The relation between airway pressures and lung volumes has been the focus of basic lung physiology since the mid-1800s. For review see [156](#). This relation is determined by the sum and interaction of approximately 300 million individual alveoli. Therefore, to understand the behavior of the entire lung it is helpful to look first at a single alveolus.

The membrane of each alveolus is composed of different layers, starting with the capillary endothelium, the base membranes, the connective tissue, the epithelial layer and finally the intraalveolar surfactant film. The tissue contains elastic and non elastic fibers that limit the expansion of an alveolus beyond its elastic properties. The surface tension at the air-liquid interface (see later) adds to the elastance of the alveolar wall. Thus, the surface and the tissue elements containing this surface may be thought of as acting in series [81](#), [156](#).

Figure 6 demonstrates a simplified model of alveolar expansion. During the inflation of a collapsed elastic balloon its volume and pressure are measured (A). No external restriction to its expansion is present. Initially, increases in pressure lead to little gain in volume. Once a critical opening pressure is reached, the balloon increases its volume rapidly while the pressure inside it drops. If, however, the same procedure takes place in a closed bottle (B), its expansion is limited. Beyond the opening pressure the increase in volume leads to a parallel increase in pressure. Looking at a model of four balloons with different compliance (C), the composite curve shows the opening characteristics of each individual balloon. To simulate the behaviour of the entire lung more appropriately, a mathematical model was used (D). One thousand 'alveoli' with opening characteristics similar to those of the balloons described above were used. Each individual unit had an opening pressure that was normally distributed around a mean value. The resulting graphs closely resemble standard pressure-volume curves of healthy and sick lungs.

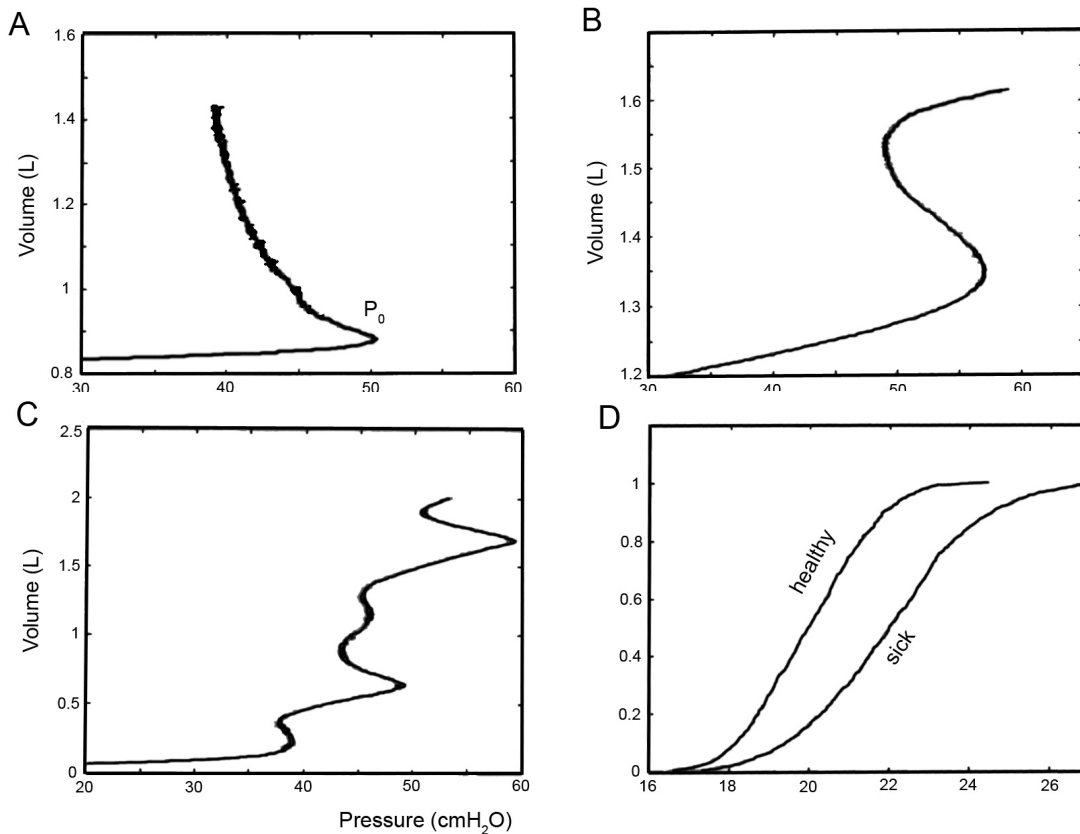


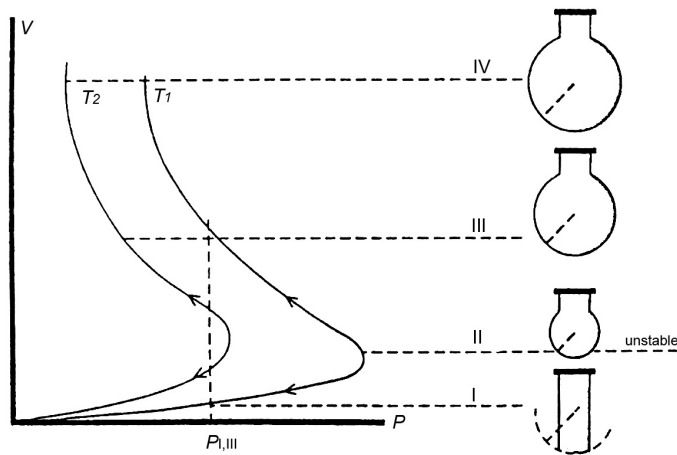
Figure 6. Experimental set-up for the simulation of alveolar recruitment using a ventilator and four rubber balloons in glass bottles. With the increase in pressure during inspiration the corresponding volume is calculated by integrating the flow signal. Pressure-volume curves are shown.

(A) A single balloon is inflated. At P_0 the critical opening pressure is reached. The balloon is recruited. Despite a reduction in pressure the increase in volume is immediate. (B) The same balloon as in A is placed into a glass bottle. Now, volume expansion is restricted, pressure increase in parallel with the gain in volume. (C) Same measurement as in B but using 4 individual balloons with different compliances. Note the four distinct points at which the balloons open . (D) Mathematical model of a pressure-volume curve. A step function was used to simulate alveolar opening. Opening pressures of 1000 alveoli were distributed normally around an assumed mean opening pressure of 20 cm H₂O in the healthy and 22 cm H₂O in the sick lung. (Provided by courtesy of Per-Göran Eriksson, Irene Lassin and Johanna Larsson at Siemens Elema, Sweden.).

The behaviour of true alveoli, however, is more complex than simply inflating elastic balloons. In 1929, Von Neergard first called attention to the contribution of alveolar surface tension to the retractive forces of the lungs [161](#). He considered the formation of a bubble on the end of a capillary tube as an analogue for the surface geometry of an alveolus [161](#) (Figure 7). For this model the law of LaPlace provides a mathematical explanation: $P = 2\gamma / r$, where P is the pressure inside the bubble, γ the surface tension of the liquid and r the radius of the bubble. Before any pressure is applied, the fluid covers the orifice of the capillary tube as a flat perpendicular film. Increasing the pressure in the capillary will start the formation of a small bubble. The pressure within the system rises until the bubbles shape approaches that of a hemisphere. The bubble now has the same radius as the capillary. Once the pressure within the bubble exceeds a critical pressure, the bubble overcomes the hemispheric state; it opens. Now, the bubble can be kept open with a much lower pressure than the critical opening pressure (Figure 7). In an open bubble the pressure changes required to induce certain changes in volume are now significantly lower compared to the closed state [156](#).

Applying these concepts to the inflation of a surfactant-deficient collapsed alveolus, it becomes apparent that surface forces, as stated in the law of LaPlace, act predominantly at a low alveolar radius. They hinder alveolar opening. Once, however, the alveolus is opened, and while maintaining the identical opening pressures the volume increases rapidly to about 2/3 of maximal volume up to the point where the tissue forces begin to oppose further expansion. The pressure within this newly expanded alveolus can now be decreased until the bubble reaches its unstable state, and collapses [147](#).

In a healthy alveolus with a normal surfactant system this collapse pressure is reduced to 3-5 cm H₂O. In other words, due to the fact that at end-expiration surface tension decreases almost to zero, the required pressure to stabilize healthy alveoli is only 3-5 cm H₂O, which is equal to the existing transpulmonary pressure. This, in general, prevents a healthy lung from end-expiratory collapse. However, should the alveolus collapse, an active re-expansion (as stated above) is required to open it [150](#), [162](#), [163](#). Thereafter, the pressures are reduced and kept at a value slightly above the previously determined collapsing pressure. This pressure level depends mainly on the function of the surfactant system [2](#), [76](#), [164](#).

**Figure 7.**

Physiological behaviour of the alveolus. The pressure-volume (P-V) relation is shown on the X-Y axes. On the right side, the status of the broncho-alveolar unit; its radius (r) reflects the P-V relation (I-IV). Surface tension in pathological (T_1) and normal conditions (T_2) is shown. The arrows indicate the direction from closed (bottom) to open (top) states and vice versa (*modified from 2*).

In summary, the behaviour of alveoli is quantal: they are either open or closed [165](#). No stable state in between these endpoints exists. This quantal alveolar physiology was demonstrated by Mead and Staub [156](#), [166](#) and was recently confirmed in computer tomography studies by Wegenius et al. [167](#), [168](#).

The need to open the lung

The three subsequent statements by Lachmann and colleagues describe the treatment concept in words and in Figure 8 [2](#), [150](#):

1. One must overcome a critical opening pressure during inspiration
2. This opening pressure must be maintained for a sufficiently long period of time
3. During expiration, no critical time that would allow closure of lung units should pass.

Years later, Slutsky's consensus document on mechanical ventilation indirectly confirms these ideas by claiming that the prevention or reversal of atelectasis is one of the clinical treatment objectives of mechanical ventilation [169](#), [170](#).

From this statement it is concluded that, it is obligatory to 'open up the lung and keep the lung open' [150](#). The Open Lung Concept defines the conceptual goals of this treatment strategy which is accomplished by a predetermined sequence of therapeutic phases, each with its specific treatment objective [2](#), [150](#), [162](#), [163](#). Figure 9 depicts these different phases schematically.

As shown in Figure 9, the goal of the initial increase in inspiratory pressure is to recruit collapsed alveoli and to determine the critical lung opening pressure. Then, the minimum pressures that prevent the lung from collapse are determined. Finally, after an active re-opening maneuver sufficient pressure is implemented to keep the lung open.

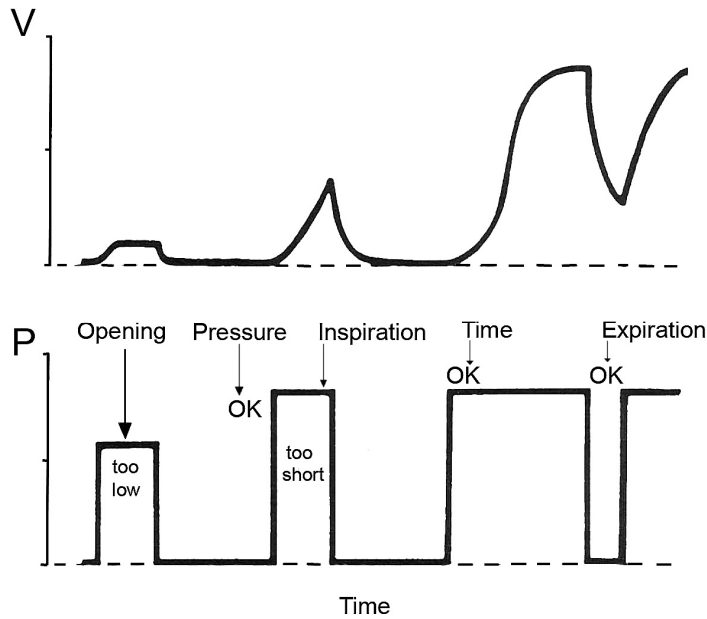


Figure 8. Schematic diagram showing the improvement of tidal volume (V) during pressure-controlled ventilation of surfactant-deficient lungs, in relation to variations in inflation pressure (P), and inspiratory time. Note: Choosing an expiratory time which is too short to empty the lung prevents expiratory collapse and results in autoPEEP.

Procedure to open the lung

In the following paragraphs instructions for the clinical application of the method are provided. For details see Figures 10-12. All interventions discussed below are safe only when used with a pressure-control mode of ventilation; their application with a volume-control mode may even be considered a professional error [150](#), [165](#). Before opening the lungs, in adult patients the end-expiratory alveolar pressure is set between 15 and 25 cm H₂O either as static or as auto PEEP, or as a combination of both. This level will be sufficient to keep those alveoli, which are to be recruited by the peak inspiratory pressures, open [171](#), [172](#).

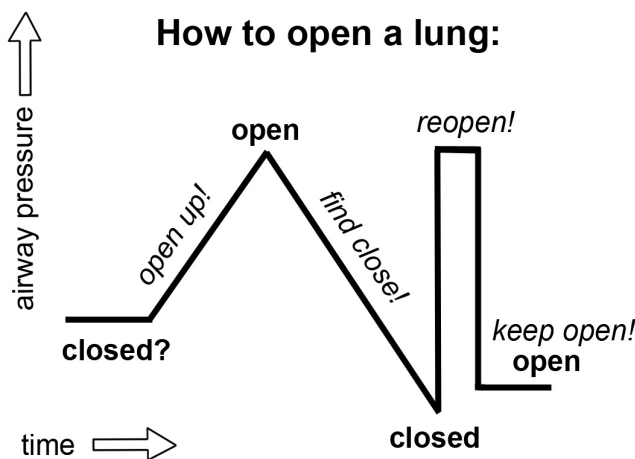


Figure 9. Schematic representation of the opening procedure for collapsed lungs. Note: The imperatives (!) mark the treatment goal of each specific intervention. The **bold** words mark the achieved state of the lung. At the beginning the precise amount of collapsed lung tissue is not known.

Gattinoni et al., using CT scan imaging, have shown that PEEP markedly reduces the fraction of lung that undergoes tidal reopening and closure during mechanical ventilation of patients with ARDS and that the pressures required to open up all alveoli, especially the ones in the dependent lung, are very high [171](#).

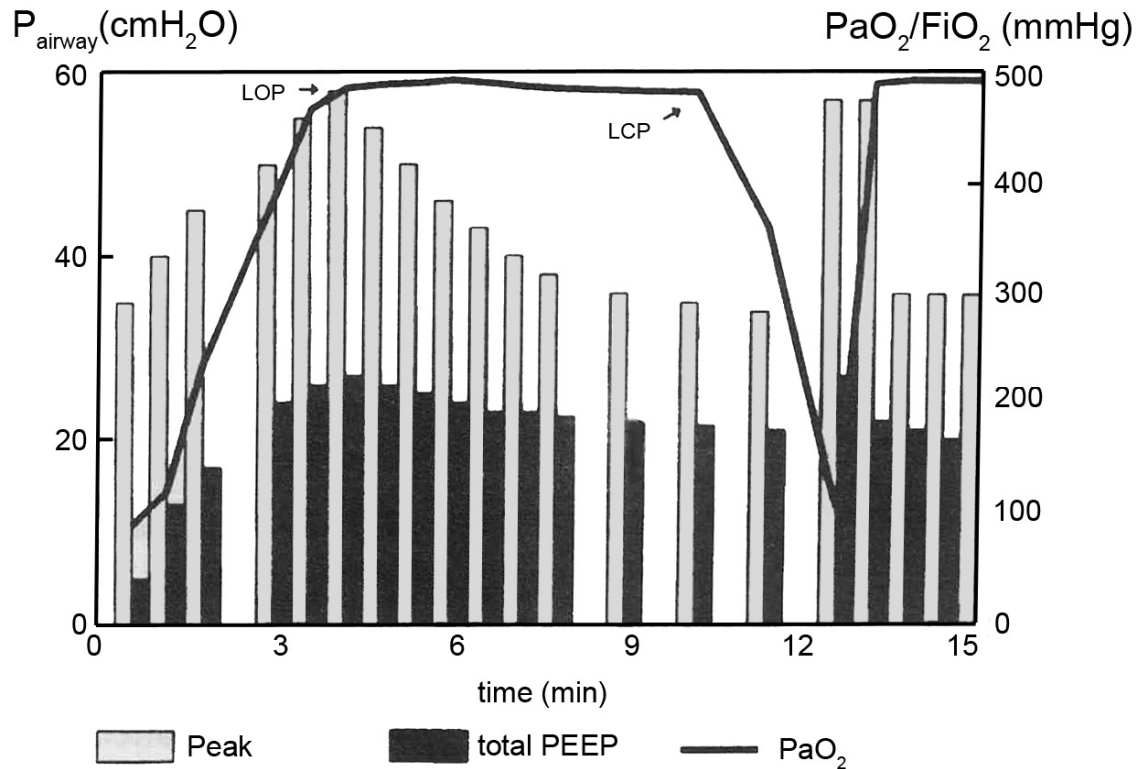


Figure 10. Schematic representation of the opening procedure for atelectatic lungs. $\text{PaO}_2/\text{FiO}_2$, peak inspiratory pressure (PIP) and total PEEP as a result of the I/E ratio and the respiratory rate are displayed over time on the abscissa. $\text{PaO}_2/\text{FiO}_2$ increases in parallel with increases in airway pressure. At a certain airway pressure, referred to as lung opening pressure (LOP) the lung is opened fully, indicated by a $\text{PaO}_2/\text{FiO}_2$ of almost 500 mm Hg. $\text{PaO}_2/\text{FiO}_2$ stays high despite a reduction of PIP and total PEEP until pressures reach a critical value where lung areas collapse, referred to as lung closing pressure (LCP). $\text{PaO}_2/\text{FiO}_2$ falls immediately. Full recruitment is achieved, again, by setting the airway pressures to the opening values determined before. Airway pressures are then reduced to and maintained at values above the previously determined closing pressures. $\text{PaO}_2/\text{FiO}_2$ remains high because the lung is open.

Their studies show that using peak pressures of 50 cmH_2O were insufficient to fully recruit alveoli in the dependent parts of the lung and/or PEEP pressures as high as 20 cmH_2O were insufficient to keep the recruited alveoli fully open in patients with moderate to severe respiratory failure [171](#). Peak pressures are successively incremented in steps of 3 to 5 $\text{cm H}_2\text{O}$ until a peak airway pressure between 45 and 60 $\text{cm H}_2\text{O}$ is reached. During the process of opening the lungs the PaO_2 helps to guide this effort, because it is the only parameter that reliably correlates with the amount of lung tissue that participates in gas exchange [150, 153, 162, 163, 172, 173](#). In severe lung disease, frequent measurements of arterial blood gases are required during this titration process. Even better is the use of on-line blood gas measurements (Figure 12). A more than proportional increase in the size of the tidal volume following the increase in airway pressure or sometimes in inspiratory time also indicates successful alveolar recruitment (Figure 8) [173-176](#). If the lung disease is inhomogeneous, which is almost always the case [177](#), there may

be a large difference in the pressures needed to open collapsed alveoli; some have always been open while others need further increments in pressure to overcome their closed state [171](#), [172](#), [178](#), [179](#). This may result in a transient hemodynamic compromise since open lung units expand further and compress the adjacent capillary bed [2](#), [165](#). Therefore, it is important during the opening procedure to maintain a sufficient intravascular volume. That is why it may even be necessary to administer fluids or to give inotropic support to the right heart before one can finalize the opening procedure. This type of hemodynamic support will be superfluous at lower airway pressures. If a further increase in airway pressure does not result in a parallel increase in PaO₂, peak inspiratory pressures can carefully be reduced. During this phase the PaO₂ should, however, remain high despite the reduction in airway pressure until the critical level of pressure is reached at which the least compliant parts of the lung start to collapse [2](#), [150](#), [180](#), [181-184](#). Should this occur, the inspiratory pressure is immediately set back to the previously determined opening pressure and is kept there for a short period of 5 to 10 seconds [2](#), [184](#). The lung tissue is yet again fully recruited, and the peak inspiratory pressures should then be reduced to a level which is safely (usually 2 cm H₂O) above the closing pressure. If, however, no collapse occurs, the reduction of peak inspiratory pressure continues until alveolar ventilation becomes too low to remove carbon dioxide effectively. PaCO₂ increases beyond normal limits. At this point PEEP is reduced, too.

As stated before, at the beginning of the opening procedure, PEEP was set to a relatively high value of 15-25 cm H₂O to prevent collapse of recruited alveoli. It is, however, not known if this PEEP is really necessary. Therefore, the same procedure as described above for the peak inspiratory pressure, is now performed to find the lowest level of PEEP. After having opened the lungs, again, the inspiratory and expiratory pressures are set to levels above the closing pressures and are kept there until the lung condition changes. In the further course of the disease the ventilator can be adjusted carefully to any changes in the patient's respiratory condition. A reduction of the total level of support is generally possible after a successful alveolar recruitment [165](#). It is important to realize that the lung has to be kept open at all times [150](#), [181-183](#).

Should a renewed collapse of alveoli occur, often caused by intrapulmonary suction or disconnection, a fall in PaO₂ indicates that a recruitment maneuver has to be performed in the same way as previously described. Also later in the weaning phase, one has to guarantee a sufficient level of PEEP which keeps the entire lung open. This can be combined either with pressure or volume support to ensure an adequate CO₂ removal. Both levels of support should be reduced according to the improvement of the patients condition.

After opening the lung and finding the lowest pressures to keep it open, the resulting pressure

amplitude is minimized and at the same time pulmonary gas exchange is maximized. The therapeutic steps of the The Open Lung Concept help define a ventilatory condition which saves the lung from further damage, allows a reduction of FiO_2 , promotes the resorption of interstitial and intrapulmonary edema, and also leads to a reduction of the pulmonary artery pressures by overcoming hypoxic pulmonary vasoconstriction.

Oxygenation and Alveolar Collapse

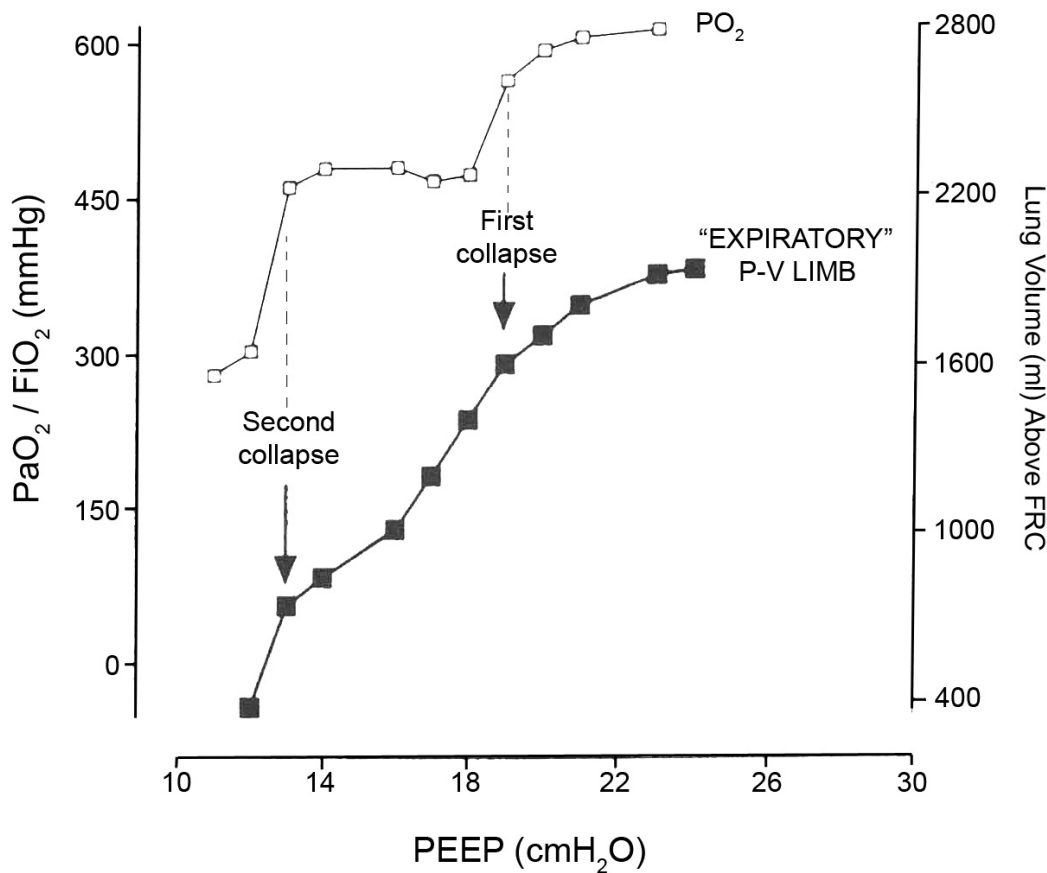


Figure 11. The deflation limb of a pressure-volume curve (open squares) together with on-line arterial oxygen tensions (solid squares) from a ventilated patient are displayed as a function of PEEP. The drop in PaO_2 corresponds with the drop in lung volumes measured by inductive plethysmography. The pronounced steps in the PaO_2 are due to the sudden alveolar derecruitment (arrows). (Figure provided by courtesy of Dr M. Amato, São Paulo, Brazil).

Earlier data ² and recent data by our group ¹⁸⁵ suggest that when applied early during induction of ALI in rats, this open lung approach prevents a decrease in pulmonary compliance associated with modes of ventilation that do not maintain an open lung during induction of ALI, and suggest that it is necessary to apply this open lung strategy early during the course of ALI/ARDS ^{2, 185}. A clinical study by Amato et al. showed that a ventilation strategy aimed at

opening atelectatic lungs and keeping them open at all times in combination with a treatment strategy of permissive hypercapnia and a restriction on the size of tidal volume and limited peak inspiratory pressures, resulted in a higher rate of weaning from mechanical ventilation, lower rate of barotrauma, and improved 28 day survival in ARDS patients compared to conventional ventilation [186](#). In a preliminary report the authors stratified the patients according to PEEP levels and concluded that PEEP levels higher than 12 cmH₂O and especially higher than 16 cmH₂O significantly improved survival of these ARDS patients [187](#).

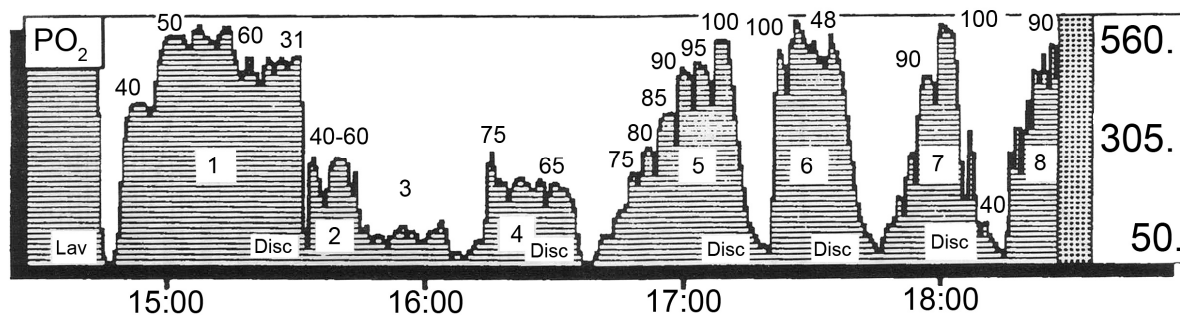


Figure 12. Original registration of on-line blood gases (Paratrend 7, Diametrix, UK) of an experimental animal model of acute respiratory failure. The lungs of a pig were lavaged and ventilated in a volume controlled mode with PEEP of 5 cm H₂O, tidal volume of 15 ml/kg BW and a respiratory rate of 15 bpm, obtaining a PaO₂ < 50 mmHg at an FiO₂ = 1.0. Thereafter, the lung was opened by a peak airway pressure of 40, 50 and 60 cm H₂O successively, which resulted in a PaO₂ above 500 mmHg (1). After this opening-up procedure, the lungs could be kept open at a peak airway pressure of 31 cm H₂O with a total PEEP of 18 cmH₂O (1). The animal was then disconnected and the alveoli were left to collapse. Peak inspiratory pressures of 40-60 cmH₂O were now insufficient to re-open the alveoli (2). During period (3) several attempts were made to treat the sick lung with more conventional ways of mechanical ventilation, which all failed to restore arterial oxygenation. Thereafter, an increase up to 75 cmH₂O peak inspiratory pressure, followed by a peak inspiratory pressure of 65 cmH₂O with total PEEP levels of 15-20 cmH₂O, was not able to restore arterial oxygenation to > 500 mmHg at an FiO₂ = 1.0 (4). During period (5) the lung was step-wise recruited by increasing peak inspiratory pressure up to 100 cmH₂O. A PaO₂ > 500 mmHg was achieved with full lung recruitment. The animal was then disconnected again from the ventilator. In period (6), The lung was recruited with a peak airway pressure of 100 cmH₂O with a total PEEP of 24 cmH₂O, and the lung could be kept open with a peak inspiratory pressure of 48 cmH₂O and a total PEEP of 21 cmH₂O thereafter. The animal was disconnected again, fully recruited at a peak pressure of 100 cmH₂O, but peak pressure was then reduced to 40 cmH₂O, which was insufficient to keep the lung open. Period (8) indicates the start of a new recruitment maneuver after the last disconnection.

Note: Repeated disconnection of a sick lung results in a more severe stage of respiratory failure which requires higher opening pressures (compare period (1) with periods (6-7)).

Alveolar recruitment should almost always be possible during the first 48 hours on mechanical ventilation (which may be more difficult if the disease exists for a longer period of time [188](#)). Even if not all of the lung tissue may be fully recruited for gas exchange, as in consolidating pneumonia, this ventilatory strategy will prevent further damage to the re-aerated part of lung [154](#), [72](#), [73](#), [157](#), [189](#). Not every patient with respiratory failure requires an invasive form of respiratory support with prolonged inspiratory times, high levels of PEEP and peak airway pressures. Also for patients with milder or even no form of respiratory dysfunction, it is imperative to adjust the ventilator such that further progression or the generation of lung disease is avoided. Also, for

this purpose the lung has to be opened. The ventilatory strategy has to achieve that which patients normally do: they take deep breaths, they sigh and stretch to re-aerate non-ventilated parts of the lung. All considerations for the very sick lung as were mentioned above are, without exception, also valid for the milder forms of disease; only the level of support is lower in the latter cases.

Increasing airway pressures to levels of around 40 cm H₂O for 2-3 minutes will recruit normal alveoli that became atelectatic when the patient was lying in the supine position, underwent anesthesia and surgery [176](#), or was disconnected from the ventilator [163, 173-175, 178](#). When all lung units have been reopened, the necessary inspiratory pressures can be reduced to levels that assure adequate tidal volume. The PEEP level should never be below 5 cm H₂O since all recruited alveoli have to be kept open during expiration.

Summary

The basic treatment principles are:

- **Open up the whole lung with the required inspiratory pressures**
- **Keep the lung open with PEEP levels above the closing pressures**
- **Maintain optimal gas exchange at the lowest mean airway pressure to minimize hemodynamic interference**

With the strict application of these principles, a prophylactic treatment is at hand that is aimed at preventing ventilator-induced lung injury and pulmonary complications. A typical performance of an open lung management is presented in Table 1.

Ventilatory support techniques directed at an open lung

Conventional mechanical ventilation or High frequency oscillatory ventilation?

HFOV is intended to counterbalance the increased tendency for collapse by applying a constant distending pressure ('mean airway pressure') [184](#). However, In the first HFOV trial, a low distending airway pressure was used in order to minimize the risk of barotrauma [190](#). Due to the limited success of the early HFOV trials [190](#), exogenous surfactant therapy with conventional mechanical ventilation (CMV) is now routinely used in most neonatal ICUs to prevent and treat neonatal RDS [191](#). It has been shown that avoiding large pressure-volume variations with HFO does not totally prevent lung injury if HFO does not maintain lung volume [192](#), which can be achieved by a preliminary sustained static inflation to recruit the greatest possible number of lung units before starting HFO [183](#). Such recruited alveoli should be kept open during the whole respiratory cycle [150](#).

Table 1. Representative data from a patient who responded as expected to the Open Lung Management

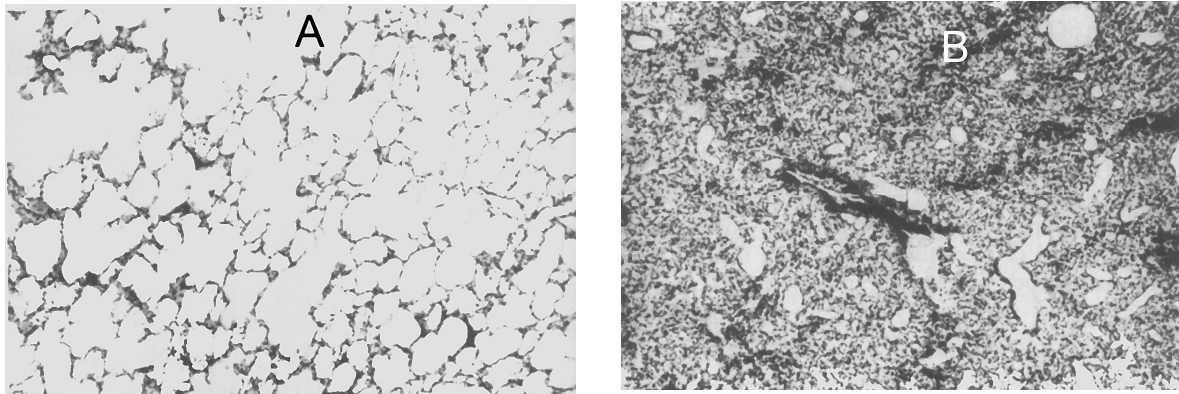
Patient at day 2: volume control ventilation with tidal volumes of 6 ml/kg	$FiO_2 = 70\%$ $PEEP = 11 \text{ cm H}_2\text{O}$ $RR = 32, I:E = 1:2$ $TV = 6\text{ml/kg} = 480\text{ml}$ $PAWP = 26 \text{ cm H}_2\text{O}$ $SaO_2 = 92\%$ $PaO_2 = 72 \text{ mm Hg}$ $PaCO_2 = 48 \text{ mm Hg}$	Slight hypoventilation
Switch to pressure controlled ventilation FiO_2 set to 100% $I:E = 1:1, RR: 40$ $PAW 26 \text{ cm H}_2\text{O}$ $PEEP 20$	$SaO_2 = 100\%$ $PaO_2 = 140 \text{ mm Hg}$ $PaCO_2 = 39 \text{ mm Hg}$	
Increase PAWP to 45 cm H ₂ O for 3 breaths (5 seconds), then back to 30	$PaO_2 = 265 \text{ mm Hg}$	
1 min after stabilization increase PAW to 50 for 5 sec., then back to 30	$PaO_2 = 350 \text{ mm Hg}$	
1 min after stabilization increase PAWP to 55 for 5 sec., then back to 30	$PaO_2 = 530 \text{ mm Hg}$ $PaCO_2 = 28 \text{ mm Hg}$	
1 min after stabilization increase PAWP to 60 for 5 sec., then back to 30	$PaO_2 = 531 \text{ mm Hg}$ $PaCO_2 = 28 \text{ mm Hg}$	PaO_2 does no further increase Opening pressure = 55 cm H₂O
Decrease PAWP to 28	No changes	
Decrease PAWP to 26	No changes	
Decrease PAWP to 24	No changes	
Decrease PAWP to 23	$PaO_2 = 480 \text{ mm Hg}$	The lung is collapsing Minimum required upper pressure limit = 24 cm H₂O
Recruit the lung (PAWP to 55, then 24)		Reach opening pressure and set back just above minimum required upper pressure limit
PAWP 24, decrease PEEP to 18	No changes	
PEEP to 17	$PaO_2 = 541 \text{ mm Hg}$	
PEEP to 16	$PaO_2 = 470 \text{ mm Hg}$	Minimum required lower pressure limit = 17 cm H₂O
Set PEEP to 17 Increase PAWP to 55 for 5 sec (recruit) and back to 24	$PaO_2 = 544 \text{ mm Hg}$ $PaCO_2 = 31 \text{ mmHg}$ $VCO_2 = 480 \text{ ml/min}$	Optimal CO ₂ removal requires VCO ₂ of 430 ml/min
Increase RR to 47, I:E = 3:2	$PaCO_2 = 36 \text{ mm Hg}$ $VCO_2 = 435\text{ml/min}$ $TV = 395 \text{ ml} = 4.9 \text{ ml/kg}$	Smaller pressure amplitude due to auto-PEEP and increased dead space ventilation
Decrease FiO_2 to 30%	$PaO_2 = 114 \text{ mm Hg}$ $PaCO_2 = 36 \text{ mm Hg}$	Optimized patient ventilation at open lung

However, as discussed above alveoli should be actively opened and a relatively high mean airway pressure has to be used to stay above the closing pressure to avoid hypoxemia and lung injury [182, 192](#). The application of this concept in HFO is called the high-lung volume strategy and results of recent pilot studies in neonates with RDS applying this strategy are encouraging [192-194](#). This does not mean that we need HFOV to adequately ventilate surfactant deficient lungs. A recent study by our group demonstrated that pressure controlled CMV combined with The Open Lung Concept can achieve the same levels of oxygenation (>500 mmHg) and carbon dioxide exchange (35-45 mmHg) as HFO in surfactant deficient lungs, with the same low levels of protein infiltration as long as the open lung concept is applied in both CMV and HFO. There are now more studies that support the concept, that not the mode of ventilation is important, but that the concept behind every ventilator strategy comply with the rules of the open lung concept [195, 196](#). A recent study by our group compared the use of HFOV with the use of CMV combined with surfactant in the treatment of acute lung injury caused by normal saline lavage in adult rabbits. Arterial oxygenation could be kept >350 mmHg in both groups during 4 hours by using appropriate MAwP. Deflation stability was assessed by decreasing mean airway pressure from 12 to 9 to 6 cmH₂O and measuring PaO₂ at 10 min intervals. During deflation stability, mean PaO₂ values decreased to post-lavage values in the HFOV group after MAwP was decreased to <9 cmH₂O but not in the CMV group that received surfactant. To evaluate lung injury, we assessed lung histopathology and the lungs were fixed at 6 cmH₂O at the end of the experimental period. The results are shown in Figure 13. The HFOV group showed more cellular infiltrate and epithelial damage compared with the surfactant-treated CMV group. This demonstrates that even using appropriately high enough mean airway pressures with HFOV can not prevent ventilation-induced lung injury and that surfactant is necessary to prevent it [197](#).

Role of exogenous surfactant

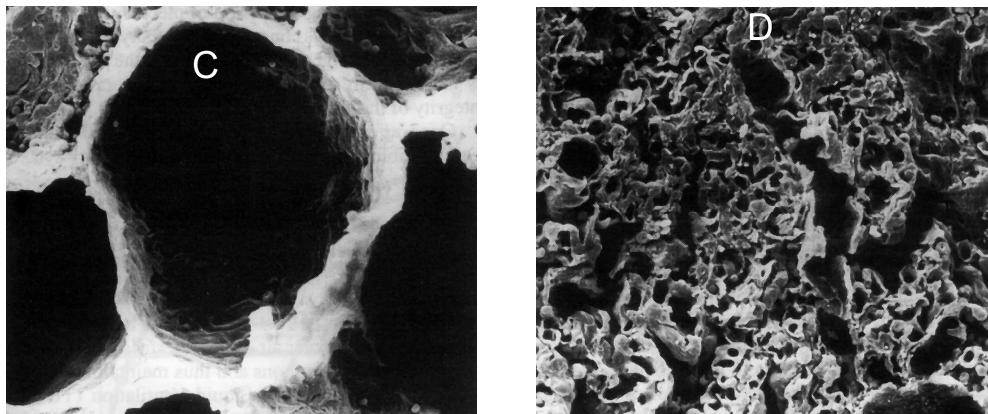
Until now, only a few studies have been published on the combined use of surfactant and HFOV in animals or humans [158, 192, 194-199](#). It was shown that after surfactant therapy HFOV was superior to CMV in improving pulmonary function and reducing lung injury [97, 158, 192, 194-198](#). In these studies, however, HFOV was used in combination with the high-lung volume strategy whereas CMV was not. Recently, Froese and colleagues [158](#) compared HFOV to CMV after surfactant therapy at low and high-lung volume and confirmed that HFOV at high-lung volume was superior to the alternatives in improving gas exchange and lung mechanics in lung-lavaged rabbits. Surprisingly, these authors were not able to maintain oxygenation above 350 mmHg (according to the high-lung volume strategy) after surfactant therapy with the use of CMV [158](#).

Figure 13. Lung histopathology of lung lavaged rabbits treated with CMV combined with surfactant and HFOV (with courtesy of Dr. R Schnabel, Dept. Pathology, Ruhr University Bochum, Germany).



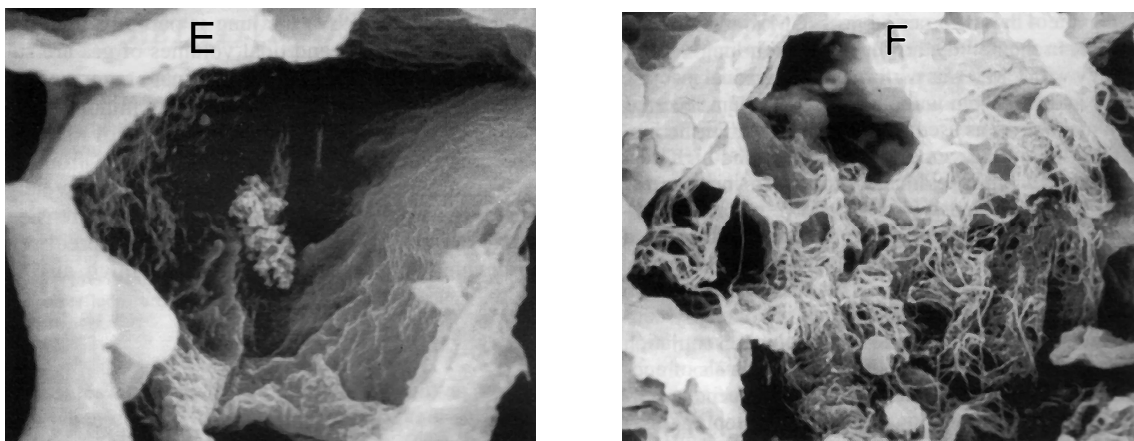
(A) Aerated lung tissue with inconspicuous alveolar walls (surfactant group). Light microscopy, hematoxylin-eosin stain; magnification 100x

(B) Atelectasis of the lung tissue (hfo group). The alveolar walls are thickened by an inflammatory infiltrate and an edema. Light microscopy, hematoxylin-eosin stain; magnification 100x



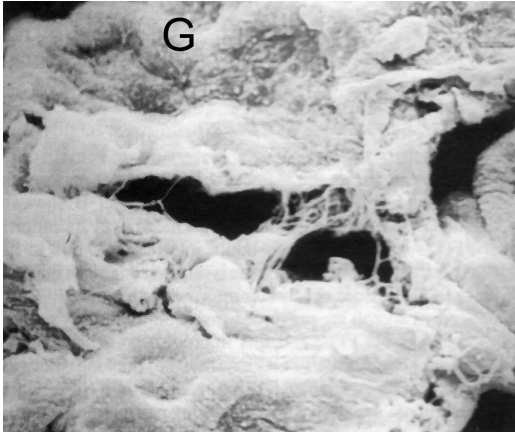
(C) Alveoli with normal diameter and inconspicuous alveolar walls (surfactant group). Scanning electron microscopy.

(D) Complete atelectasis of the lung tissue (hfo group). The alveolar walls are markedly thickened. The septa contain leucocytes. Scanning electron microscopy. Same magnification as figure 15 (c).



(E) Larger magnification surfactant group. Part of an alveolus, central in the picture an exogenous surfactant particle can be seen.

(F) Same magnification hfo group. Huge networks of fibrin fibers combined with cells and hyaline membrane can be seen.



(G) Disruption of the interalveolar septum seen only in the HFO group

This is in contrast to earlier results of CMV with surfactant therapy in lung-lavaged rabbits in which oxygenation increased rapidly to prelavage values after surfactant instillation and kept stable for hours [200](#), [201](#). Froese et al. [158](#) who demonstrated that the effect of exogenous surfactant on arterial oxygenation remained stable with HFOV, whereas it decreased significantly during the 4 h study period with CMV at high-lung volume. In their study, however, the high-lung volume strategy with CMV was performed by a gradual increase of PIP and PEEP but without an active volume recruitment manoeuvre as used with HFOV [158](#). Furthermore, CMV was used with a constant flow and high tidal volumes (20 ml/kg) which is known to increase the conversion from active into non-active surfactant subfractions; this leads to a shortage of ‘active’ surfactant at the alveolar level [23](#), [25](#). Studies by our group have shown that exogenous surfactant therapy can also be optimized by conventional pressure controlled mechanical ventilation with small pressure amplitudes and high levels of end-expiratory pressure as it can with high frequency oscillation [202](#). These settings resulted in an optimal gas exchange and low levels of protein infiltration with minimal loss of active surfactant subfractions. In another study we could show that as long as the open lung concept is applied in both HFOV and CMV exogenous surfactant therapy is optimized and there is no difference in gas exchange (which is optimal with both ventilatory strategies), protein infiltration and surfactant conversion between both strategies.

Conclusion

Surfactant changes play a key role in ventilation-induced lung injury. There is a vast number of ventilatory strategies available, but all these techniques should comply with one rational concept which prevents damage due to artificial ventilation itself. It should produce minimal pressure swings during the ventilation cycle and keep the lung open during the whole ventilatory cycle. If one follows this concept, the diversity of ventilation modes may no longer cause confusion to practitioners as there is only one rational concept to preserve the integrity of the lung:

Open up the whole lung and keep it open with the least possible influence on the cardio-circulatory system.

Recent animal and clinical studies suggest that such protective lung strategies may

prevent the release of inflammatory mediators from the lung and the transfer of bacteria and bacterial endotoxins into the bloodstream and may have an important effect on mortality rates of ARDS.

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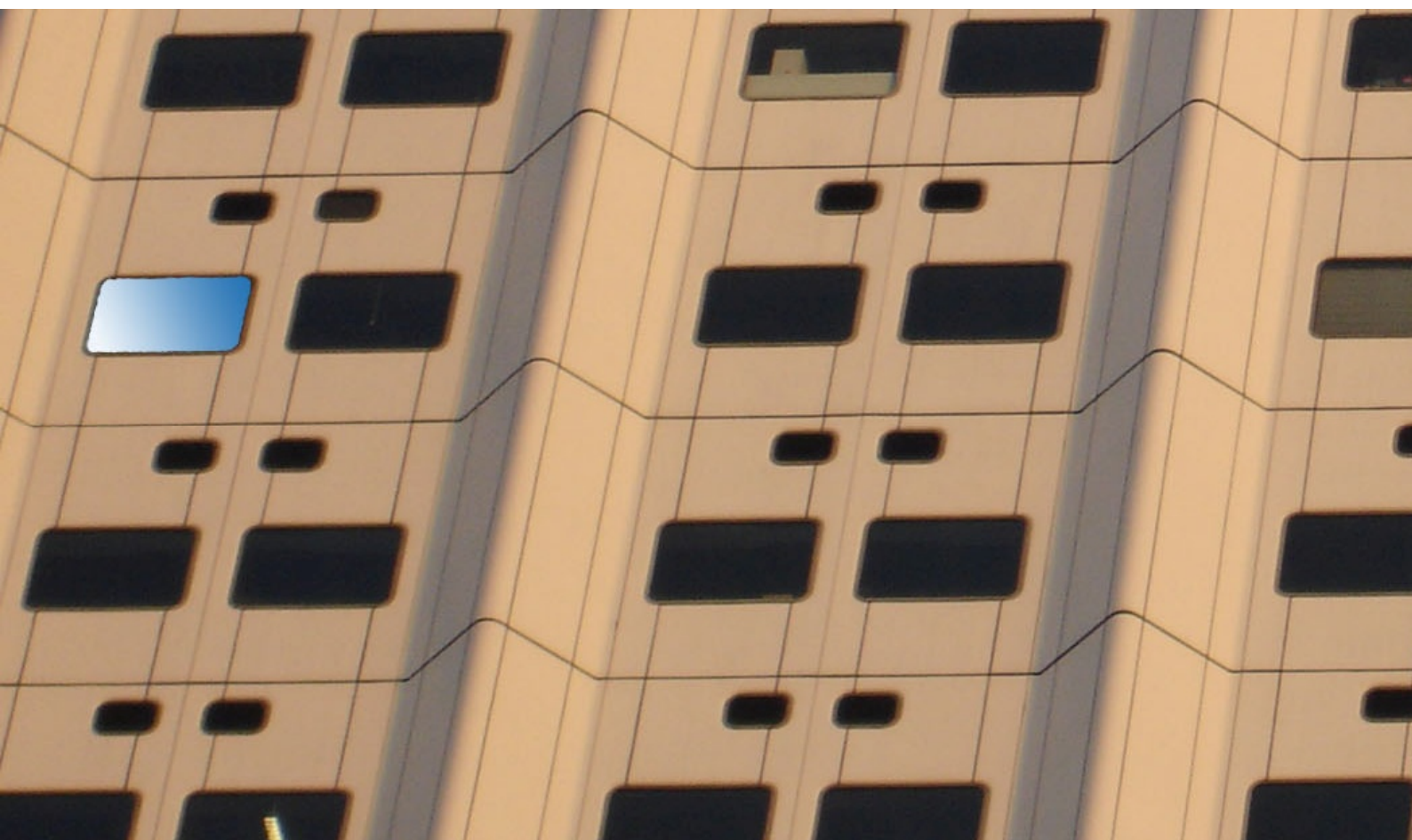
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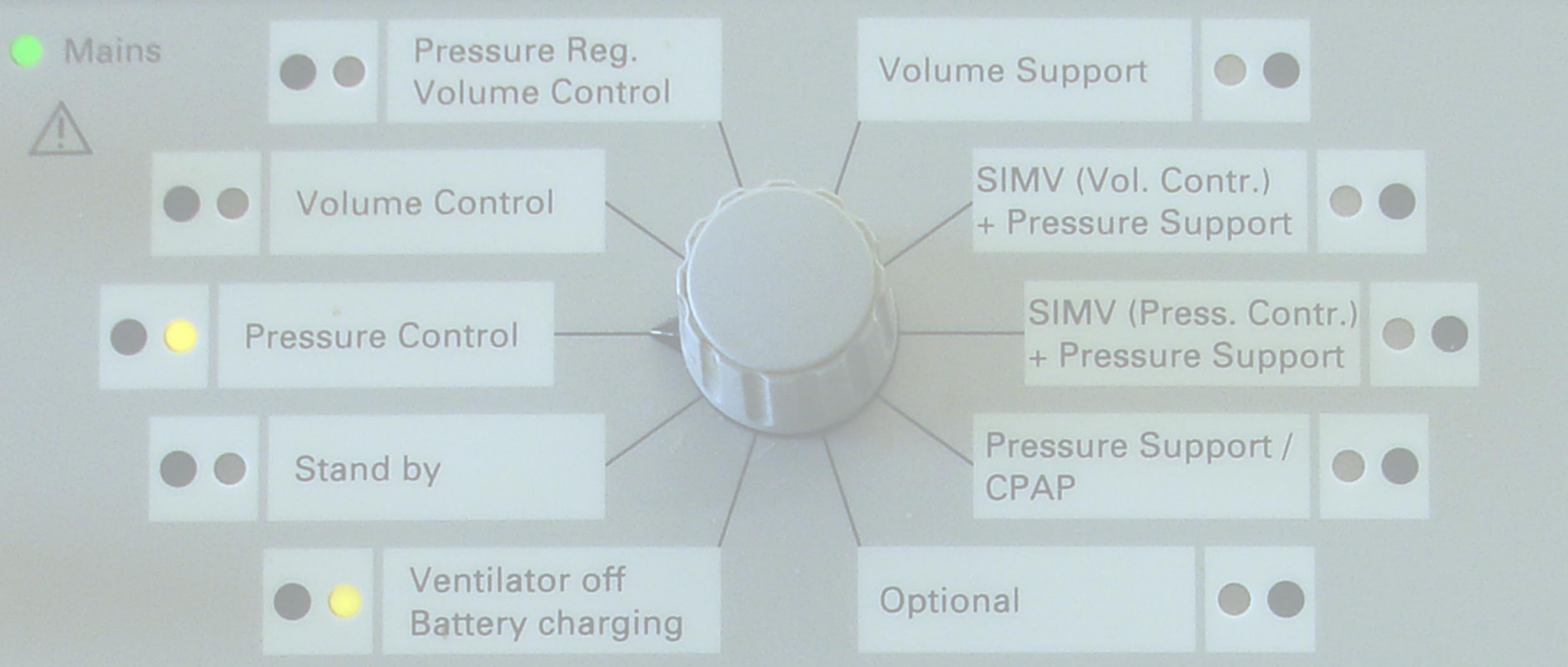
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Resp. pattern

Measured freq. b/min
50

Set freq. b/min
50

Insp. period s
0.40

Insp. flow l/s

Chapter 2

Surfactant function and exogenous surfactant therapy

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Introduction

Since 1980, more than 250,000 premature infants suffering from respiratory distress syndrome (RDS) due to a surfactant deficiency have been successfully treated with exogenous surfactant almost without any side-effects ^{1, 2}. Biochemical and biophysical abnormalities of the pulmonary surfactant system are also seen in other diseases such as the adult respiratory distress syndrome (ARDS) ^{3, 4}, infectious lung disease ⁵ and after cardiopulmonary bypass surgery ⁶. This paper focuses on the advances in surfactant replacement therapy in experimental and clinical studies.

The pulmonary surfactant system

Physiological function of the pulmonary surfactant system

Laplace, a French mathematician (1749-1827), was the first to draw attention to surface active forces in general and described the relationship between force, surface tension, and radius of an air-liquid interface of a bubble: $P = 2\gamma/r$ (P = pressure to stabilize a bubble; γ = surface tension at air-liquid interface; and r = radius of a bubble). Almost one century later, von Neergaard applied this law to pulmonary alveoli by demonstrating that the pressures required to expand an air-filled lung were almost three times that required to distend a lung filled with fluid ⁷. In this way, the surface tension effect at the air-liquid boundary was eliminated (Figure 1). From these findings, he concluded that: 1) two-thirds of the retractive forces in the lung are due to surface tension phenomenon which act at the air-liquid interface of the alveoli, and 2) the surface tension at the air-liquid interface must be reduced by the presence of a surface active material with a low surface tension to allow normal breathing ⁷.

It was not until 1959 that von Neergaard's findings became clinically relevant, when Avery and Mead published direct evidence linking absence of the surface active material to the appearance of stiff lungs in newly born premature babies with RDS ⁸. This surface active material is called pulmonary surfactant. Pulmonary surfactant is a complex of phospholipids (80-90%), neutral lipids (5-10%) and at least four specific surfactant-proteins (5-10%) (SP-A, SP-B, SP-C and SP-D), lying as a monolayer at the air-liquid interface in the lung ^{9, 10}. Surfactant is synthesized by the alveolar type II cells and secreted into alveolar spaces ⁹. The surfactant phospholipids are lying in a thin aqueous film which coats the pulmonary alveolar walls and small airways and lower its surface tension.

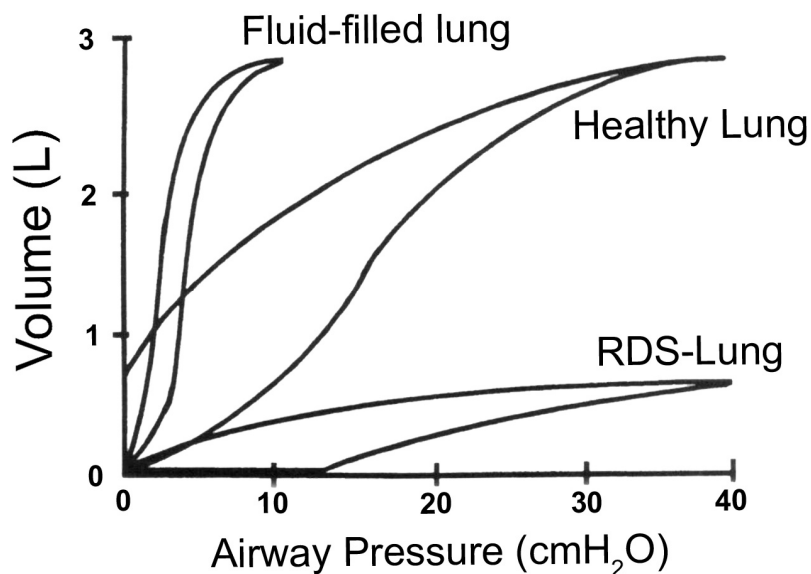


Figure 1.

P-V diagrams of a normal air-filled lung and an ARDS lung. Von Neergaard showed in 1929 that much larger pressures were required to expand an air-filled lung than a lung filled with fluid. In RDS even higher pressures are required to expand the lung, due to the high surface tension at the air-liquid interface in the alveoli caused by a diminished surfactant system.

Mechanical stabilisation of lung alveoli

The integrity of the surfactant system of the lung is a prerequisite for normal breathing with the least possible effort. The surfactant system acts by decreasing surface tension of the interface between alveoli and air. This provides an explanation as to why we have to generate a "negative" pressure by muscular effort of only 5-10 cmH₂O during each inspiration; in the absence of surfactant the surface tension at the air-liquid interface would be that of plasma and the pressure needed to maintain lung aeration would be 25-30 cmH₂O (depending on the radius of the alveoli) for which much more muscular effort would be necessary. This is a well-known problem in patients with respiratory failure [11](#).

In alveoli with different radii an equal lowering of surface tension would not, however, produce stabilization of the alveolar system. It would instead, according to the Law of LaPlace, lead to the collapse of the smaller bubbles or alveoli, and to their emptying into the larger bubbles/alveoli. Since alveoli in vivo do not exhibit such behaviour, one can conclude that the second quality of the alveolar lining layer is that it can change the surface tension in a manner related to the size of the alveoli [12](#). This may be the most important function of the pulmonary surfactant system. It will prevent end-expiratory collapse by reducing the surface tension to almost zero at small alveolar sizes.

Protection against lung oedema

Another function of the pulmonary surfactant system is stabilization of the fluid balance in the lung, and protection against edema (Figure 2) [13](#). The normal plasma oncotic pressure of 37 cmH₂O is opposed by the oncotic pressure of interstitial proteins of 18 cmH₂O, the capillary

hydrostatic pressure of 15 cmH₂O, and by the surface tension conditioned suctioning of 4 cmH₂O (Figure 2).

In general, alveolar flooding will not occur as long as the suction force in the pulmonary interstitium exceeds the pressure gradient generated by the surface tension in the alveolar air-liquid interface. Since this pressure gradient is inversely related to the radius of the alveolar curvature there is, for each combination of the interstitial resorptive force and average surface tension, a critical value for surface tension and for alveolar radius, below which alveolar flooding occurs ¹³.

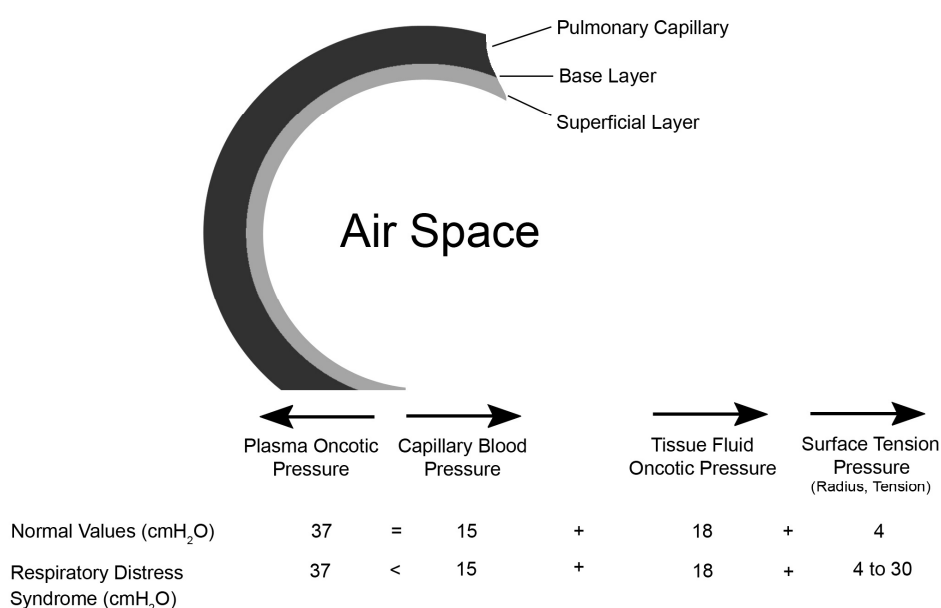


Figure 2. Simplified schematic diagram representing the factors influencing fluid balance in the lung. See text for details (from reference ²⁴).

Surfactant and airways stabilization

As early as 1970, Macklem et al. ¹⁴ called attention to the significance of bronchial surfactant for stabilization of the peripheral airways and hinted that lack of stabilization may cause airway obstruction or collapse of the small bronchi with air trapping. This has been proved in an animal model where the bronchial surfactant was selectively destroyed ¹⁵. It was demonstrated that the pressure to open up the collapsed bronchi is 20 cmH₂O.

Besides its role in mechanical stabilization, bronchial surfactant also has a transport function for mucus and inhaled particles ¹⁵. This has been proven, in vitro, in a study showing that particles on a surface film move in one direction only if the surface film is compressed and dilated, comparable to the compression and expansion during expiration and inspiration ¹⁵.

Furthermore, bronchial surfactant also acts as an antiglue factor preventing the development of large adhesive forces between mucus particles, as well as between mucus and the bronchial wall [16](#).

A further possible function of bronchial surfactant, which to date has scarcely been discussed, is its masking of receptors on smooth muscle with respect to substances which induce contraction and could lead to airway obstruction. This means that surfactant could also be possibly involved in asthma [17](#).

Surfactant and local defense mechanisms

It has also been demonstrated that surfactant plays a role in the lung's defence against infection [18](#). Surfactant, and in particular SP-A, enhances the antibacterial and antiviral defence of alveolar macrophages [18](#).

We have demonstrated that the pulmonary surfactant system may also be involved in protecting the lung against its own mediators (e.g. angiotensin II) and in protecting the cardiocirculatory system against mediators produced by the lung [19,20](#).

Functional changes due to a disturbed surfactant system

Disturbance of the surfactant system can result from different factors. Damage to the alveolar-capillary membrane leads to high-permeability edema with wash-out or dilution of the surfactant and/or inactivation of the surfactant by plasma components, such as fibrin(ogen), albumin, globulin and transferrin, hemoglobin and cell membrane lipids [21, 22](#). These components are known to inhibit pulmonary surfactant function in a dose-dependent way [22](#). Furthermore, the pulmonary surfactant may also be disturbed by the following mechanisms: breakdown of surfactant by lipases and proteases; phospholipid peroxidation by free radicals; loss of surfactant from the airways due to mechanical ventilation with large tidal volumes; disturbed synthesis, storage, or release of surfactant secondary to direct injury to type II cells [1, 21, 23](#).

Diminished pulmonary surfactant has far-reaching consequences for lung function. Independent of the cause, decreased surfactant function will directly or indirectly lead to [24](#):

1. Decreased pulmonary compliance;
2. Decreased functional residual capacity (FRC);
3. Atelectasis and enlargement of the functional right-to-left shunt;
4. Decreased gas exchange and respiratory acidosis;
5. Hypoxemia with anaerobic metabolism and metabolic acidosis; and
6. Pulmonary edema with further inactivation of surfactant by plasma constituents.

Experimental models of surfactant dysfunction and the effects of exogenous surfactant in these models

The function of pulmonary surfactant can be illustrated by studies in animal models in which the pulmonary surfactant system is impaired [25](#). As discussed below, such studies have demonstrated that exogenous surfactant instillation dramatically improves blood gases and lung mechanics [25](#). The models of surfactant deficiency in which these improvements could be demonstrated include acute respiratory failure due to *in vivo* whole-lung lavage [26, 27](#), neurogenic ARDS [28](#), respiratory failure as a result of oxygen toxicity [29, 30](#) or oxidant-producing enzymes [31](#), acute respiratory failure after instillation of hydrochloric acid [32-34](#) or plasma instillation [35](#), and respiratory failure after intoxication with N-nitroso-N-methylurethane (NNNMU) [36](#) or paraquat [37](#) and many others for review [38](#) and Table 1. We will now go into more detail on some of these animal models.

In vivo lung lavage

Experiments on post-mortem lung specimens have revealed that a considerable portion of the alveolar phospholipids can be recovered by repeated washing of the airspaces [67](#). With this knowledge a model of ARF was developed by Lachmann and colleagues for use in different animals, in which pulmonary surfactant is removed by repeated bi-lateral whole lung lavage with warm saline [68, 69](#).

Table 1. Animal models of surfactant impairment

Model		Animals	Source
<i>Immature newborn</i>		Rabbits	Lachmann 39,40
<i>Animals</i>			Nilsson 41
		Lambs	Ikegami 42, 43
<i>Neurogenic</i>	Head injury	Cats	Crittenden 44
<i>ARDS</i>	Vagotomy	Rats	Goldenberg 45
			Berry 46
<i>Oxygen</i>		Different	Klein 47
<i>toxicity</i>		species	Cochrane 48
			Ennema 49
<i>Oxygen producing</i>	Xanthine Oxidase	Guinea pigs	Saugstadt 50
<i>enzymes</i>			Lachmann 31
<i>Septicemia</i>	E. Coli/E. Coli	Rats	Oldham 51
		endotoxin	Rinaldo 52
		Pseudomonas	Mustard 53
		aeruginosa	Dogs Hanly 54
<i>NNNMU</i>		Rats	Harris 36
		Rabbits	Lewis 55
		Dogs	Ryan 56
<i>Oleic acid</i>		Rabbits	Hall 57
		Sheep	Zelter 58
<i>Paraquat</i>		Mice	Manktelow 59
		Rats	Robertson 60
<i>Hemorrhagic shock</i>		Dogs	Henry 61
		Baboons	Moss 62
<i>TNF</i>		Guinea pigs	Stephens 63
<i>BAL with plasma</i>		Dogs	Nieman 64
<i>Bile acid aspiration</i>		Rabbits	Kaneko 65
<i>VILI</i>		Rats	Haitsma 66

In these lavage models, severe respiratory insufficiency is defined as a decrease in PaO₂ to below 80 mmHg during artificial ventilation with 100% oxygen. The first lavage already results

in a significant reduction of thorax-lung compliance and repeated lavage results in a derangement of lung function persisting for at least 8 h. These disturbances, which include a 35% decrease in functional residual capacity (FRC), are almost certainly secondary to increased surface tension in the alveolar lining [68-71](#), especially since lung lavage with saline does not alter elastic properties of the pulmonary parenchyma [70](#). Removal of alveolar surfactant by in vivo lung lavage significantly increases permeability of the alveolo-capillary membrane, as demonstrated by Wollmer and colleagues in their studies on ^{99m}Tc -DTPA clearance [72](#). Histologic examination of lungs from animals 5 min after the last lavage shows atelectasis, desquamation of bronchial and bronchiolar epithelium, and incipient formation of hyaline membranes [68](#). Electron microscopic studies of the same lungs revealed necrosis and desquamation of alveolar type II cells. Some type II cells were also necrotic, but the majority of these cells seemed to be intact and should thus be available for synthesis and discharge of surfactant phospholipids [68](#). These changes are likely due to the combination of surfactant deficiency and mechanical ventilation.

Intratracheal instillation of exogenous surfactant in lavaged lungs results in dramatic improvements of gas exchange, even when the treatment is given 2 hours after the lavage procedure [26, 68, 73-75](#). Improved blood gases were stable for at least 5 h [75](#), whereas in lavaged animals receiving no surfactant PaO_2 remained low despite ventilation with PEEP and pure oxygen. Histologic sections from surfactant-treated animals showed a uniform pattern of well-aerated alveoli with only minimal intra-alveolar edema and hyaline membranes, whereas animals receiving no surfactant, ventilated with the same ventilator settings, had extensive atelectasis and prominent hyaline membranes [26, 75](#).

The lung lavage models are useful for a variety of experimental purposes, including testing of different surfactant preparations [26, 38, 74, 76](#) and studies on the significance of different ventilator settings [69, 77](#). The lavage model was found suitable to test the influence of anti-surfactant monoclonal antibodies on the ability of exogenous surfactant to restore gas exchange [78](#).

Pulmonary infection

Pneumonia is an important cause of respiratory failure and is associated with increased alveolar permeability leading to pulmonary edema, hemorrhage and atelectasis [79, 80](#). The pathophysiological changes in pneumonia include hypoxemia, decreased FRC, decreased total lung capacity (TLC), decreased lung compliance, and a diminished surfactant system [5, 81, 82](#).

As far back as 1964, Sutnick and Soloff [79](#) demonstrated that the surface tension of BAL

fluid from lung tissue with pneumonia was increased; they suggested that the pulmonary surfactant became inactivated and was responsible for atelectasis. It has since been demonstrated that the surfactant system is also impaired in bacterial [5](#), [82](#) and viral pneumonia [83](#), as well as in *Pneumocystis carinii* pneumonia [84](#), [85](#).

In bacterial pneumonia surface tension of BAL fluid is increased, whereas SP-A content and total surfactant lipids are all significantly decreased [5](#). In viral pneumonia, Stinson *et al.* [83](#) demonstrated that pulmonary surfactant activity is decreased; these workers suggested that injury and destruction of type II pneumocytes by the virus was the cause of reduced surfactant activity. Recently, two studies have demonstrated surfactant abnormalities in HIV positive patients with *Pneumocystis carinii* pneumonia [84](#), [85](#). In these patients qualitative and quantitative changes were seen in the surfactant composition, as well as increased phospholipase A₂ activity [85](#). There is increasing evidence that especially the surfactant B protein plays an important role in the immune defence mechanism of the lung [86](#).

Bacteria, bacterial toxins, viruses, phospholipases, and proteinases released from inflammatory cells interact either directly with the surfactant film (Figure 3), or damage the endothelial and epithelial cells leading to high permeability edema [5](#). It is well established that plasma proteins of the edema fluid inactivate the surfactant [21](#), [22](#). Due to the decreased surfactant activity, surface tension at the alveolar walls increases, leading to increased suction forces across the alveolar-capillary membrane [87](#). This finally results in a vicious circle [88](#).

Evidence thus exists of a deficiency of active pulmonary surfactant in patients with pneumonia and this would be the rationale for exogenous surfactant therapy. The effectiveness of surfactant therapy was demonstrated in different animal models suffering from viral pneumonia or *Pneumocystis carinii* pneumonia [89-91](#). In viral pneumonia, tracheal administration of exogenous surfactant led to improved lung compliance and improved FRC [89](#), as well as to restoration of gas exchange [90](#).

Similarly in rats with *Pneumocystis carinii* pneumonia, surfactant instillation led to an improvement in blood gases [91](#). The therapeutic dosage in all three experimental studies was 200 mg surfactant/kg. Song *et al.* [92](#) recently confirmed the rapid effect of surfactant therapy (160 mg/kg) on arterial oxygenation in rats with *E.coli* pneumonia. These results show that there was a shortage of functional alveolar surfactant in these animals with infected lungs and that these models may be suitable to test surfactant function.

Exogenous surfactant is known to affect various macrophage functions including stimulation of superoxide production, which enhances phagocytosis of bacteria and viruses [93](#).

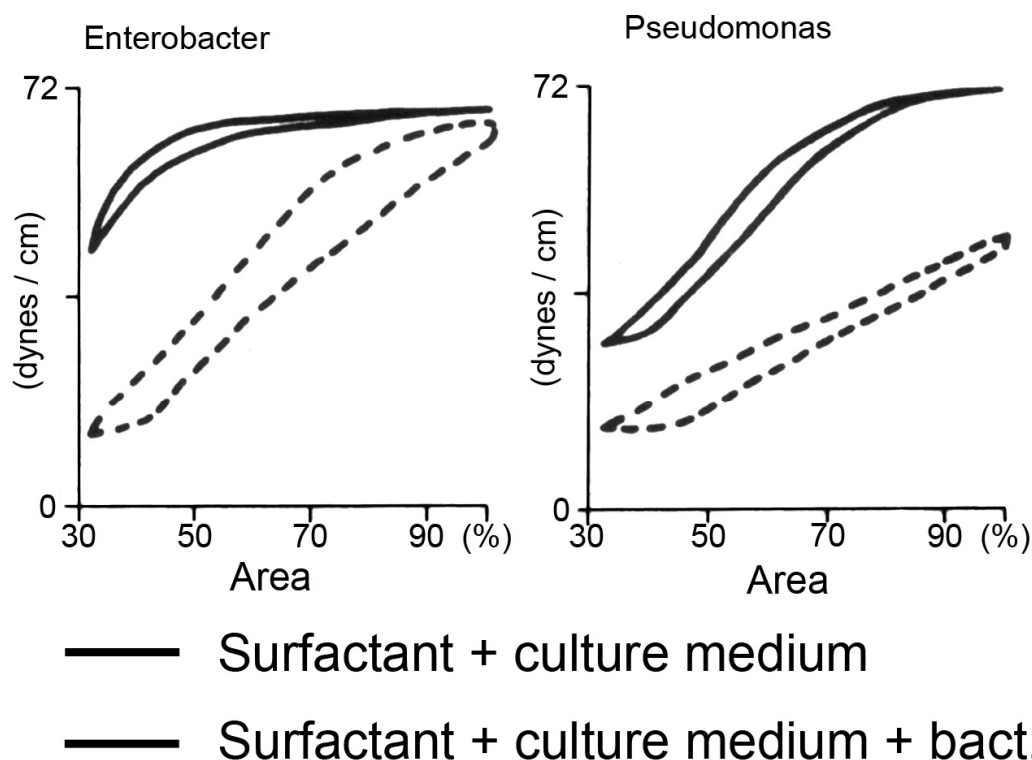


Figure 3. Surface tension (dynes/cm) vs. area for surfactant mixed with medium compared with surfactant mixed with medium and bacteria after incubation of 24 h. Surface tension is increased when surfactant is mixed with a bacterium. The surface tension is measured with a Wilhelmy balance.

Hydrochloric acid instillation

The pulmonary effects of acid aspiration have been extensively documented in animal models [94-96](#). Data from these studies show that in the pH range below 2.5, the degree of lung injury is proportional to the hydrogen ion concentration of the aspirated material (for review see [97](#)). Hydrochloric acid (HCl) aspiration leads to damage to the alveolo-capillary membrane with subsequent increased permeability to and accumulation of plasma-derived proteins. It is also likely that surfactant is directly damaged by HCl. Shortly after acid instillation, type II pneumocytes show cytoplasmic swelling [95](#) and the surface properties of alveolar lavage fluid and lung extracts are characterized by an increased minimal surface tension and decreased hysteresis [98, 99](#).

Several studies have been performed on animals suffering from respiratory failure due to HCl aspiration and treated with surfactant. Kobayashi and colleagues demonstrated that surfactant instillation could only partially restore gas exchange in rabbits suffering from respiratory failure due to HCl aspiration after intra-alveolar lung edema was removed by BAL; surfactant, when given without prior lung lavage, only prevented further deterioration of blood gases [32](#). Lamm and co-workers showed improved lung distensibility in rabbits receiving surfactant 5 min after HCl aspiration, but no effect was seen on blood gases [100](#). It has been

shown that intratracheal surfactant instillation within 10 min after HCl aspiration could prevent development of respiratory failure, whereas surfactant, given after deterioration of gas exchange, had no effect on pulmonary gas exchange [33](#). In another study we demonstrated that lung function could be restored in rats suffering from respiratory failure due to HCl aspiration after lavaging the lungs with a diluted surfactant suspension, or by lavaging the lungs with saline, followed by surfactant treatment [101](#).

Because surfactant treatment alone does not suffice to improve pulmonary function it is speculated that BAL with saline (followed by surfactant treatment), or with a diluted surfactant suspension, removes inhibitory proteins present in the alveolar edema fluid, resulting in improved efficacy of surfactant treatment. Other studies have shown that these proteins inhibit the function of the surfactant system dose-dependently; they demonstrated that approximately 1 mg surfactant phospholipids is required to overcome the inhibitory effect of approximately 1 mg plasma proteins [22](#).

Immunological mechanisms

Monoclonal antibodies

Inactivation of the small (15 kDa) hydrophobic surfactant proteins decreases surfactant function with a subsequent loss of pulmonary function, resulting in ARDS. Suzuki and co-workers [102](#) intraperitoneally inoculated mice with hybridoma cells, producing a monoclonal antibody against the 15 kDa surfactant protein. Subsequently, animals developed respiratory failure within 9 days with markedly reduced lung-thorax compliance, lung congestion, alveolar collapse, hemorrhagic pulmonary edema and hyaline membranes, mimicking ARDS. The importance of this surfactant-associated protein for surfactant function was also demonstrated in a surfactant replacement study using the lavage model in rats. It was shown that inhibition of an 11 kDa surfactant protein caused loss of effectiveness of the used surfactant preparation [78](#). Kobayashi and co-workers demonstrated that as a result of inactivation of surfactant by a monoclonal anti-surfactant antibody against Sp-B, this preparation was not able to improve lung compliance in immature rabbit fetuses [103](#). In another study Robertson and colleagues demonstrated that intratracheal instillation of a monoclonal antibody against Sp-B in near-term newborn rabbits caused a decrease in lung-thorax compliance, when compared to rabbits which received nonspecific IgG [104](#). These studies demonstrate that Sp-B is important for surfactant function and inhibition of this protein in a surfactant preparation makes the preparation inactive or maybe less active.

Anti-lung serum

The structural and functional pulmonary abnormalities due to capillary leakage in ARDS can be imitated in experimental animals by administration of anti-lung serum [105-107](#). This serum contains antibodies against surfactant-associated proteins and against the basement membranes of alveolar capillaries.

Earlier, Lachmann and colleagues demonstrated in artificially ventilated guinea pigs, that intravenous injection of anti-lung serum leads to respiratory failure with decrease in lung compliance, hypoxemia, and respiratory and metabolic acidosis. Morphologic lesions include interstitial and intra-alveolar edema, hemorrhage, and desquamation of alveolar epithelium. These structural abnormalities are associated with a marked reduction in phospholipid content of the lungs [108](#). The decrease in lung compliance reflects accumulation of fluids in the air spaces and inactivation of surfactant components by plasma proteins. Instillation of a single dose of exogenous natural surfactant (280-350 mg/kg BW) after deterioration of blood gases improved gas exchange.

Surfactant in asthma models

In an asthmatic attack, increased mucus secretion, transudation of proteinaceous fluid and mucociliary disturbance causes mucus plug formation [109](#). As long as 23 years ago, Macklem and co-workers [14](#) concluded that the existence of bronchial surfactant is a prerequisite for normal lung function and that disturbance of the bronchial surfactant leads to airway obstruction and impaired bronchial clearance. It is tempting, therefore, to speculate on the possible role of surfactant in reversing airway obstruction in asthma attack [110](#). Furthermore, it has been demonstrated that beta-adrenergic agents and glucocorticoids, which are two of the most widely used medications for the treatment of asthma, stimulate the release of surfactant and/or the production of surfactant [111, 112](#).

Liu *et al.* [113](#) showed that surfactant dysfunction developed in sensitized guinea-pigs challenged with aerolized ovalbumin. Surface activity of BAL from immunized, challenged animals was significantly reduced, but there was no change in the concentration nor in the composition of the surfactant phospholipids. However, the BAL fluid showed a substantial increase in the concentration of proteins, and that was the likely reason for the increased surface tension. In addition, Liu *et al.* [114](#) recently reported that repeated challenges of immunized guinea-pigs resulted in decreased synthesis and storage of pulmonary surfactant in type II cells. Further, the possibility that surfactant might play an important role in the pathogenesis of asthma was substantiated first by Lachmann *et al.* [115](#) and later confirmed by Liu *et al.* [116](#) who

showed that the increase in airway resistance, following the challenge of immunized guinea-pigs, was less prominent when surfactant had been instilled into the airway prior to the challenge.

Other mechanisms of acute respiratory failure

In a model of ventilation-induced lung injury in which tumor necrosis factor alpha production was stimulated by lipopolysaccharides, cytokine exchange between lung and blood was decreased when exogenous surfactant was applied ⁶⁶. This is extremely important because it has been documented that the lung itself can produce huge amounts of cytokines, which could be responsible for multiorgan failure and/or death ^{20, 117, 118}.

Table 1 gives an overview of other models of ARF and surfactant treatment. These will not be discussed here (For more detailed information, see Robertson and Lachmann ³⁸).

Surfactant therapy

Surfactant therapy in patients on mechanical ventilation

Postoperative Atelectasis/Pharmacological stimulation of surfactant production

Fegiz et al. reported a double-blind multicenter study to evaluate the effectiveness of ambroxol, which stimulates surfactant synthesis in the pneumocyte type II cell, to prevent postoperative bronchopulmonary complications ¹¹⁹. A total of 252 patients with chronic obstructive lung disease (COLD) undergoing upper abdominal surgery were randomly allocated to receive either 1 g/day of ambroxol intravenously for 6 consecutive days in the perioperative period or placebo. Pulmonary complications were evaluated by clinical investigations, radiographic, and blood gas analysis. There was a significant lower rate of atelectasis in the ambroxol treated group. In addition, the PaO₂ values of the ambroxol-treated group after surgery decreased less than those of the placebo-treated group from preoperative values. The treatment was well tolerated, although nausea was significantly more frequent in the ambroxol-treated group ¹¹⁹.

These data indicate that ambroxol, but also possibly surfactant therapy, should be considered as an alternative and new pharmacologic approach for the prevention of postoperative pulmonary complications.

Acute Lung Failure/ALI/ARDS

As discussed previously, in 1967 Ashbaugh and co-workers ¹¹ described 12 adult patients with acute respiratory failure which did not respond to usual therapy. The common pathological characteristic of ARDS is increased capillary permeability associated with damage of the

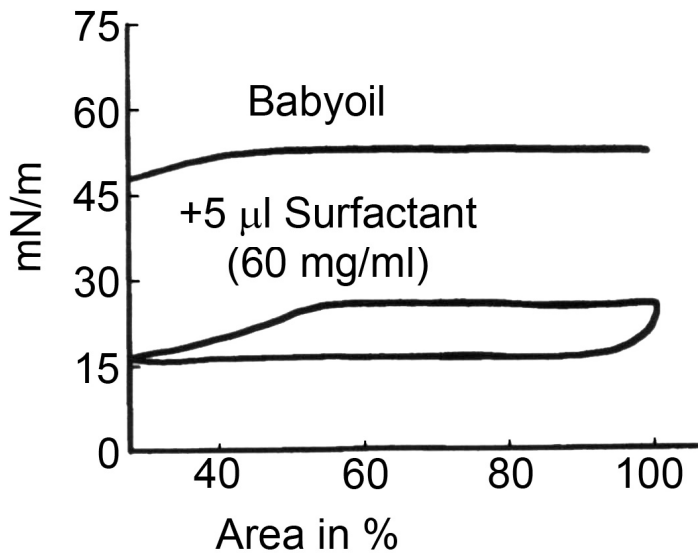
alveolar epithelium. From what is discussed above, it is clear that the progress of the disease depends on the balance between new production and release of surfactant into the alveoli and its inactivation/loss from the alveoli and airways due to factors such as mechanical ventilation and protein inactivation. If the synthesis is reduced e.g. by influenza virus, hypoxia or hyperoxia, etc., an imbalance between new synthesis and demand will result. This will finally lead to a total loss of functional active surfactant, resulting in failure of the lung as a gas exchange organ [120](#), [121](#). Thus in ARDS the surfactant deficiency is a complication of lung injury rather than, as in neonatal RDS, a primary etiological factor [122](#), [123](#). Studies on exogenous surfactant in neonates have shown a clear reduction of mortality and morbidity [124](#). Despite increased sophistication in methods of respiratory support, mortality associated with ARDS currently remains between 48 and 75%, depending on the etiology [125-128](#). Nowadays, it is more appropriate to speak about the Acute, rather than Adult, Respiratory Distress Syndrome (ARDS), since ARDS is not limited to adults [129](#).

Analyses of lung surfactant recovered in BAL from patients with ARDS, or from animal models of acute respiratory failure, demonstrate disturbances of the lung surfactant system [121](#), [130](#). Reduction of surfactant activity is associated with increased minimal surface tension of lung extracts or lung homogenates, and compositional changes of surfactant and/or decreased surfactant content of the lungs [120](#), [121](#), [131-133](#). Ashbaugh and colleagues [11](#) were the first to demonstrate decreased lung compliance and decreased surface activity in lung extracts from two ARDS patients. Since then, several studies have demonstrated qualitative and quantitative changes of surfactant in BAL fluid from ARDS patients [120](#), [121](#), [134](#), [135](#). Recently, Gregory and colleagues [136](#) demonstrated that several of these alterations already occur in patients at risk of developing ARDS, suggesting that these abnormalities of surfactant occur early in the disease process.

Evidently, it is rational to administer exogenous surfactant in ARDS patients, but the question then arises why is this not yet a reality. Surfactant has been commercially available for neonates since 1987 [137](#). Surfactant therapy in patients other than neonates with RDS is almost impossible due to the fact that there is not enough surfactant available and that current prices are too high [137](#).

Lachmann was the first to treat a non-neonatal patient with exogenous surfactant in 1983 and this was published a few years later [138](#). Lachmann *et al.* [139](#) treated a four-month-old boy with exogenous surfactant who had aspirated baby oil and developed acute respiratory failure. In this case, the question was raised whether the detergent effect of the baby-oil could be overcome by administering exogenous surfactant.

SURFACE TENSION

**Figure 4.**

Surface pressure (mN/m) vs area for baby oil and baby oil with surfactant, measured with a Wilhelmy balance (from reference [139](#)).

Using a Wilhelmy balance, it was first demonstrated that surfactant decreased surface tension despite the presence of baby-oil (Figure 4). Therefore, a natural surfactant (250 mg/kg; 40 mg/ml) was instilled intratracheally in this patient and arterial oxygenation was measured with an intra-arterial PO₂ catheter. Figure 5 shows that arterial oxygenation improved from 150 to 300 mmHg within two hours, despite a decrease of the peak airway pressure. Richman *et al.* [140](#) studied three adult patients with ARDS of different etiologies. These patients were treated with 4 g of a natural surfactant (60 mg/kg), delivered bronchoscopically in divided doses to each lobe. After surfactant instillation, arterial oxygenation improved immediately in two patients, but sustained for only one hour. In the third patient, arterial PO₂ improved over time and persisted throughout the observation period (72 hours). No significant changes were seen in compliance or functional residual capacity (FRC) in any of these patients. Joka and Obertacke [141](#) treated an 18-year-old victim of a motorcycle accident with a post-traumatic ARDS. Directly after exogenous surfactant instillation, PaO₂ improved, whereas mean pulmonary arterial pressure, FiO₂ and alveolar protein leakage in BAL were decreased. The surfactant was given in two doses (on days 9 and 13 after the accident), with a total concentration of about 50 mg/kg body weight. Nosoka and co-workers [142](#) demonstrated improvement in PaO₂ and chest X-ray after multiple instillations of surfactant in two adult patients with ARDS.

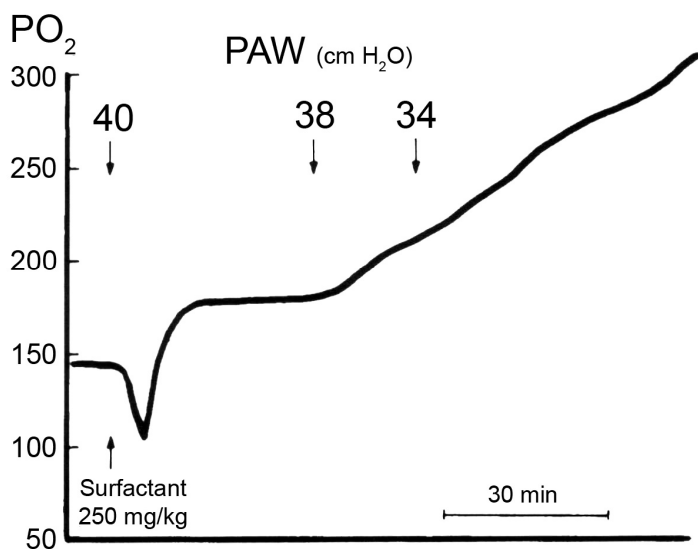


Figure 5.

Effect of surfactant on arterial oxygenation in a child who had aspirated baby oil and developed acute respiratory failure. PO_2 was measured continuously with an intra-arterial PO_2 catheter. The boy was mechanically ventilated with pressure controlled ventilation 100% oxygen, 70% inspiration time, frequency 45/min Peak airway pressure (PAW) of 40 cmH_2O and a PEEP of 5 cmH_2O . After surfactant instillation, peak pressure could first be reduced to 38 cmH_2O and 30 minutes later to 34 cmH_2O (from reference [129](#)).

The first patient received surfactant 20 times (240 mg each) during 38 days, whereas the second patient received three doses of surfactant (also 240 mg each) on three consecutive days (antibodies to the natural surfactant were not detected in either patient). Stubbig *et al.* [143](#) reported a case of surfactant therapy in a 21-year-old man who developed ARDS after severe lung contusion due to a car accident. No improvement occurred during conventional ventilatory treatment, including inverse-ratio ventilation and high-frequency ventilation. After instillation of a natural surfactant (on day 15 after the accident; 38 mg/kg) followed by some additional interventions, they observed there was a progressive improvement in PaO_2 and chest X-ray, whereas FiO_2 , inspiration time and PEEP level could be reduced. Marraro *et al.* [144](#) treated two adolescents who developed ARDS which appeared during leukemia treatment with surfactant (patient one, 60 mg/kg and patient two, 40 mg/kg). Arterial oxygenation improved within three hours (patient one: 60 to 350 mmHg; patient two: 160 to 300 mmHg) during mechanical ventilation with 100% oxygen. Haslem and colleagues [145](#) treated four adult patients with late stage of ARDS with a single bolus of artificial surfactant (75 mg/kg) and found no sustained clinical improvement. In contrast to the results of Haslem and co-workers [145](#), Heikinheimo *et al.* [146](#) reported successful treatment of a 50-year-old patient suffering from ARDS with two doses of synthetic surfactant (total amount 104 mg/kg). McBrien *et al.* [147](#) treated a nearly drowned 9-year-old boy with synthetic surfactant. PaO_2/FiO_2 was increased from 57 to 293 mmHg while PIP was reduced from 40 to 25 $cm H_2O$ and the patient was discharged successfully from the hospital 2 days later. Suzuki *et al.* [148](#) confirmed the rapid and dramatic effect of surfactant therapy on lung compliance, oxygenation and ventilation in a 3-year-old boy with refractory respiratory failure due to near-drowning. Knoch *et al.* [149](#) reported a case of

surfactant therapy in a 48-year-old patient who developed respiratory insufficiency nine days after a bicycle accident. The left lung could not be ventilated even after separate artificial ventilation of each lung. After administration of a bolus of synthetic surfactant (50 mg/kg) and continued separate artificial ventilation on each side, there was a complete re-expansion of the left lung with an increase of arterial PO₂ values from 65 to 416 mmHg within a few hours (FiO₂=1.0).

Although these case reports of surfactant therapy in ARDS and infectious lung diseases showed that some patients had only a transient improvement after a single dose of surfactant, better results are seen with higher or multiple surfactant doses. This was confirmed by two pilot studies [150](#), [151](#). Gregory *et al.* [150](#) studied four different dosing strategies in 48 adults with ARDS and the results showed that maximum improvement in oxygenation, minimum ventilatory requirements, and the lowest mortality rate were obtained by using four doses of 100 mg/kg of a natural surfactant (total amount of 400 mg/kg). Walmrath and colleagues [151](#) reported an impressive acute improvement of arterial oxygenation in response to bronchoscopic application of a large quantity of natural surfactant (300 mg/kg) in 27 adult patients with severe ARDS and sepsis. In 7 of their patients, a second dose (200 mg/kg) was required within 24 h to achieve a prolonged effect on gas exchange. In contrast to these results, Anzueto *et al.* [152](#) demonstrated that administration of aerosolized artificial surfactant had no effect on mortality and lung function in a multicenter, randomized placebo-controlled trial in 725 patients with sepsis-induced ARDS. The authors speculated that one of the reasons for the lack of response could be that less than 25 mg surfactant per kg body weight was actually delivered into the lungs due to the method of administration, which is only one-sixteenth of the dosage used by Gregory and colleagues [150](#).

Thus, the reason for lack of response or only transient improvement after exogenous surfactant application in patients with ARDS has been attributed to the inhibition of the instilled surfactant by plasma components filling the alveolar space [22](#). Therefore, the therapeutic goal must be to overcome the inhibitor capacity by large amounts of exogenous surfactant. This implies that if after surfactant instillation there is no, or only transient, improvement of blood gases in these patients (fibrotic lungs excluded), this does not mean that surfactant treatment does not work but only that the concentration of the exogenous surfactant used is too low in relation to the amount of surfactant inhibitors in the lung. As discussed previously, we have demonstrated in rats that approximately 1 mg surfactant phospholipids is required to overcome the inhibitory effect of 1 mg plasma proteins [22](#).

Secondly, it was shown that for an optimal effect of exogenous surfactant therapy, the

surfactant proteins SP-B and C are essential ¹⁵³. A recent study comparing a synthetic surfactant preparation with a natural derived surfactant preparation in neonates with respiratory distress syndrome clearly demonstrated a significant lower predischarge mortality in infants that received the natural derived surfactant ¹⁵³. This may also explain why a multicenter study with a surfactant containing only a recombinant surfactant protein C failed to show a beneficial effect on lung function and mortality in patients with pneumonia/aspiration ^{154, 155}. In contrast, Lachmann conducted a multicentre, open, randomised, parallel, prospective, phase II study with an animal-derived surfactant to evaluate primarily the effect on lung function for 7 days after the last surfactant application and secondarily on mortality at day 28. They enrolled 36 adult ALI/ARDS patients with a PaO₂/FiO₂ ratio of 180 ± 85 at a PEEP of 11.8 ± 3.8 cmH₂O. 22 patients were treated with natural-derived exogenous surfactant from pig lungs and 14 patients got standard therapy. Patients included in the study should not be ventilated for more than 60 h. There were no statistical differences in any measured lung function parameter between the two study groups. However, there was significantly less mortality in the surfactant-treated patients (9.1%) compared with the patients who received standard therapy (42.8%) ¹⁵⁶.

Pneumonia

A few preliminary reports indicate that instillation of exogenous surfactant might be efficacious in patients with infectious lung diseases. Lachmann ¹³⁸ has treated a 4-year-old patient with a bacterial pneumonia and acute respiratory failure. Surfactant was instilled three times in succession (150; 100; 50 mg/kg) and, after the last dose of surfactant, gas exchange improved dramatically. Chest X-ray taken four hours after surfactant instillation showed nearly 'normal' lungs. Buheitel *et al.* ¹⁵⁷ reported exogenous surfactant therapy in two patients with acute respiratory failure due to viral pneumonia. In the first patient, 5.5 months old, gas exchange improved immediately after tracheal instillation of a natural surfactant (300 mg/kg) but was sustained only for three hours. A second dose was given (215 mg/kg) 12 hours after the first one, arterial oxygenation improved slowly over time and after five weeks the boy could be extubated and recovered. The second patient was almost four years old and was pressure-controlled ventilated as follows: peak airway pressure of 41 cm H₂O, PEEP of 12 cm H₂O, I/E ratio of 2:1 and FiO₂ of 0.95. Blood pressure decreased several times before surfactant instillation, probably as a result of the high ventilator pressures. After a natural surfactant (50 mg/kg) was instilled, arterial oxygenation improved immediately and peak pressure could be reduced from 41 to 30 cm H₂O. However, six hours after surfactant instillation the patient died, probably as a result of cardiovascular failure. Putz *et al.* ¹⁵⁸ confirmed the successful treatment

of ARDS caused by viral pneumonia in a 3-year-old boy with a bolus of a natural surfactant (200 mg/kg). Slater *et al.* ¹⁵⁹ reported an infant with *Pneumocystis carinii* pneumonia associated ARDS who failed to respond to standard therapy, including corticosteroids, but improved dramatically with artificial surfactant (40 mg/kg).

Mikawa *et al.* ¹⁶⁰ showed an improvement of oxygenation after *selective* instillation of exogenous surfactant in a 71-year-old man who developed lobar bacterial pneumonia and unsatisfactory oxygenation following abdominal surgery. On post-operative day five, surfactant was instilled via a bronchofiberscope which enabled deposition of a small amount of surfactant in the infected lobe only. This method of instillation was probably chosen due to the prohibitive price of surfactant and the non-availability of sufficiently large amounts of surfactant for use in adults. Immediately after surfactant application, oxygenation increased; this improvement was not dramatic but this may be attributed to the low dose of surfactant (240 mg) given. One may speculate that if surfactant had been administered to the whole right lung, the increase in oxygenation would be more striking.

The reported experimental and clinical findings support the role of exogenous surfactant therapy in bacterial, viral and *Pneumocystis carinii* pneumonia. Pneumonia and ARDS are closely associated. Not only is ARDS often complicated by nosocomial infections, but infection can also lead to ARDS ¹⁶¹.

Surfactant and patients following cardiopulmonary by-pass

It has been seen that cardiopulmonary by-pass (CPBP) causes atelectasis, low pulmonary compliance, decreased diffusing capacity, and pulmonary hemorrhage ¹⁶². Various explanations have been proposed including a loss or inhibition of surfactant. do Campo and colleagues ⁶ measured the total phospholipid concentration of BAL fluid of patients immediately after cardiac surgery with CPBP and found a significantly lower concentration of total phospholipids than in normal patients. Furthermore, radiological pictures taken immediately post-operatively appeared similar to ARDS ⁶.

In this respect, the same investigators treated these patients with nebulised exogenous surfactant and found improved arterial oxygenation ¹⁶³. The magnitude of improvement was higher in the group treated with 30 mg/kg than in the group receiving 10 mg/kg. Strüber *et al.* ¹⁶⁴ reported good result of surfactant therapy in a 38-year-old patient with severe lung failure after CPBP. Treatment was first started with inhaled nitric oxide at a concentration of 30 ppm and arterial PO₂ increased from 50 to 160 mmHg with 100% oxygen. However, lung compliance continued to drop and 2 days later, gas exchange deteriorated in spite of nitric oxide

inhalation. Then, a bolus of exogenous synthetic surfactant was applied, resulting in an increase of compliance from 18 to 35 ml/mmHg and gas exchange improved from 70 to 220 mmHg.

During spontaneous breathing - Chronic Obstructive Pulmonary Disease (COPD) (Cystic Fibrosis (CF), Asthma)

Cystic Fibrosis

It has been reported that lipids, and particularly total phospholipids, are increased in CF compared with normal airway secretions ^{165, 166}. Moreover, Girod et al. ¹⁶⁶ have demonstrated that in CF airway secretions, rigidifying fractions of phospholipids such as sphingomyelin, phosphatidylinositol and phosphatidylserine predominate to the detriment of surface-active fractions such as phosphatidylcholine and- glycerol. This shift induces changes in the viscoelasticity as well as the surface properties of CF airway secretions. Together with mucus dehydration and hyperviscosity, this phospholipid imbalance may induce alternations in surface properties (augmentation of mucus adhesivity and stickness) which are, in turn, responsible for impairment of mucus transport and severe bronchial obstruction.

Griese and colleagues analysed the surfactant composition of bronchoalveolar lavage fluid obtained during a stable period (defined as no acute exacerbation of the lung disease, nor any changes in therapy in the 4 previous weeks) from 20 CF patients and 17 healthy children and adults ¹⁶⁷. Whereas the concentrations of total protein and phospholipids did not differ from controls, the CF patients had a reduced percentage of surface active phospholipids, phosphatidylcholine and phosphatidylglycerol, and the concentration of surfactant protein A was significantly reduced. Although the relative proportion of large aggregates was higher in CF patients, their surface active properties were inferior. This impairment of minimal surface tension was more likely due to biochemical alternations detected, than to inhibition of surfactant ¹⁶⁷. Hull and colleagues tested the hypothesis that the composition of bronchial surfactant is normal in infants with cystic fibrosis in the absence of active lung disease, but is altered by lower respiratory tract infection and inflammation ¹⁶⁸. They analysed total phospholipid (PL), disaturated phospholipid (DSP), surfactant protein-A and B (Sp-A and B) and surface activity in bronchoalveolar lavage fluid from 27 subjects with CF, of which 12 had evidence of active pulmonary infection or inflammation, and 6 infants with stridor, who served as controls. They found no differences in surfactant composition or activity between controls and patients with CF who had no active pulmonary infection/inflammation. In contrast, the DSP/PL ratio was lower in patients with CF who had pulmonary infection/inflammation than in controls and in patients with CF and no infection, whereas Sp-A concentrations were higher in this group compared to

the other two. These results suggest that bronchial surfactant of infants with CF is altered following lower airway infection/inflammation and is not a primary abnormality associated with the disorder [168](#). It may be suggested that surface-active phospholipids, such as phosphatidylglycerol may have therapeutical potential in CF by improving mucus clearability. Administration of distearyl phosphatidylglycerol (DSPG) may enable the surface-active phospholipid fraction to be restored [169](#).

Girod-de Bentzmann et al. have shown in vivo that the transport of mucus by ciliary activity and cough may be significantly enhanced by coating the mucus with liposomes of DSPG [170](#). These liposomes did not cause any toxicity to the human respiratory surface epithelial cells, suggesting that the administration of liposomes by aerosol or instillation could be a promising future strategy for the treatment of bronchial obstruction due to hypersecretion.

Griese et al. conducted a randomized cross-over double-blind pilot study on the effect of nebulized bovine surfactant versus placebo in 5 CF patients [171](#). The surfactant dose was 120 mg phospholipids or placebo aerosolized over a 30-min period for five consecutive days. The inhalation of the particles was well tolerated, and no changes in serum antibody titres against the surfactant proteins B and C were observed. However, no changes in the forced expiratory volume in one second and forced vital capacity were found before and 30 or 90 min after the inhalation. Therefore, this study showed no acute or short-term benefits of surfactant inhalation in young adults with cystic fibrosis. However, the authors concluded that a beneficial effect of exogenous surfactant could not be excluded before other reasons, such as insufficient quantity delivered, inhomogeneous distribution or inhibition of surfactant in the lungs, have been completely ruled out [172](#).

Asthma

At this moment, human data on the use of surfactant in asthma are rare. Kurishima and co-workers [172](#) showed in a pilot study of 11 patients that surfactant inhalation has a therapeutic effect in asthmatic attack. Adult patients were assigned to either placebo (=saline) or surfactant inhalation (1 ml; 10 mg/ml). After surfactant inhalation (by a hand jet nebulizer), respiratory functions markedly improved; FVC, FEV₁ and PaO₂ increased with 11.7 ± 1.3%, 27.3 ± 4.4% and 13.4 ± 0.8%, respectively. However, Bambang Oetomo *et al.* [173](#) found that inhalation of 100 mg nebulized natural surfactant did not alter airflow obstruction and bronchial responsiveness to histamine in 12 asthmatic children with severe airflow obstruction. In that study [173](#) exogenous surfactant was applied during a stable phase of the disease process and not

Table 2. Surfactant replacement therapy in clinical trials.

Postoperative atelectasis	Atelectasis ↓, PaO ₂ ↑	Fegiz 119
Acute respiratory failure/ ARDS	PaO ₂	Lachmann 139
		Richman 140
		Nosoka 141
		Stubbig 143
		Marraro 144
		Heikinheimo 146
		McBrein 147
		Walmrath 151
	PaO ₂ ↑, Mortality=,	Willson 176
	Compliance↑	Perez Benavides 177
	PaO ₂ ↑, Protein leakage↓	Joka 141
	PaO ₂ ↑, Lung compliance↑	Suzuki 148
	PaO ₂ ↑, Reexpansion lung	Knoch 149
	Mortality↓	Lachmann 156
	PaO ₂ ↑, Mortality↓	Gregory 150
	PaO ₂ =	Haslem 145
	PaO ₂ =, Mortality=	Anzueto 152
Pneumonia Bacterial	PaO ₂ ↑	Lachmann 138
		Mikawa 160
	PaO ₂ ↑	Buheitel 157
	PaO ₂ ↑	Slater 159
Cardiopulmonary bypass	PaO ₂ ↑	DoCampo 163
Thoracic aneurysm surgery	PaO ₂ ↑	Satoh 178
Cystic Fibrosis	FEV ₁ =	Griese 171
Asthma	FVC↑, FEV ₁ ↑, PaO ₂ ↑	Kurishima 172
		Bambang Oetoma 173

during an asthma attack. However, Lemarchand and colleagues [174](#) found that the bronchial clearance of DTPA is only increased in asthmatic patients during attacks but not increased in asthmatic patients in a stable phase, or asthmatics without airflow limitation but with bronchial hyperresponsiveness to metacholine. Further, it was shown that the bronchial clearance of DTPA decreased toward normal levels after recovery from the acute attack. Kurashima *et al.* [175](#) recently reported that in the acute phase of an asthma attack minimal surface tension and total protein of airway fluid increased significantly in these patients, and decreased again in the recovery phase of the attack. Further, these authors demonstrated no difference in surface activity of sputum between patients with stable asthma and healthy controls. In addition, it was shown that total phospholipids of airway fluid increased significantly in the recovery phase of

an attack, indicating an increased secretion of surfactant from type II cells. In conclusion, these results suggest that the many different facets of surfactant function in the airways need to be considered in the interpretation of the pathogenesis of bronchial asthma.

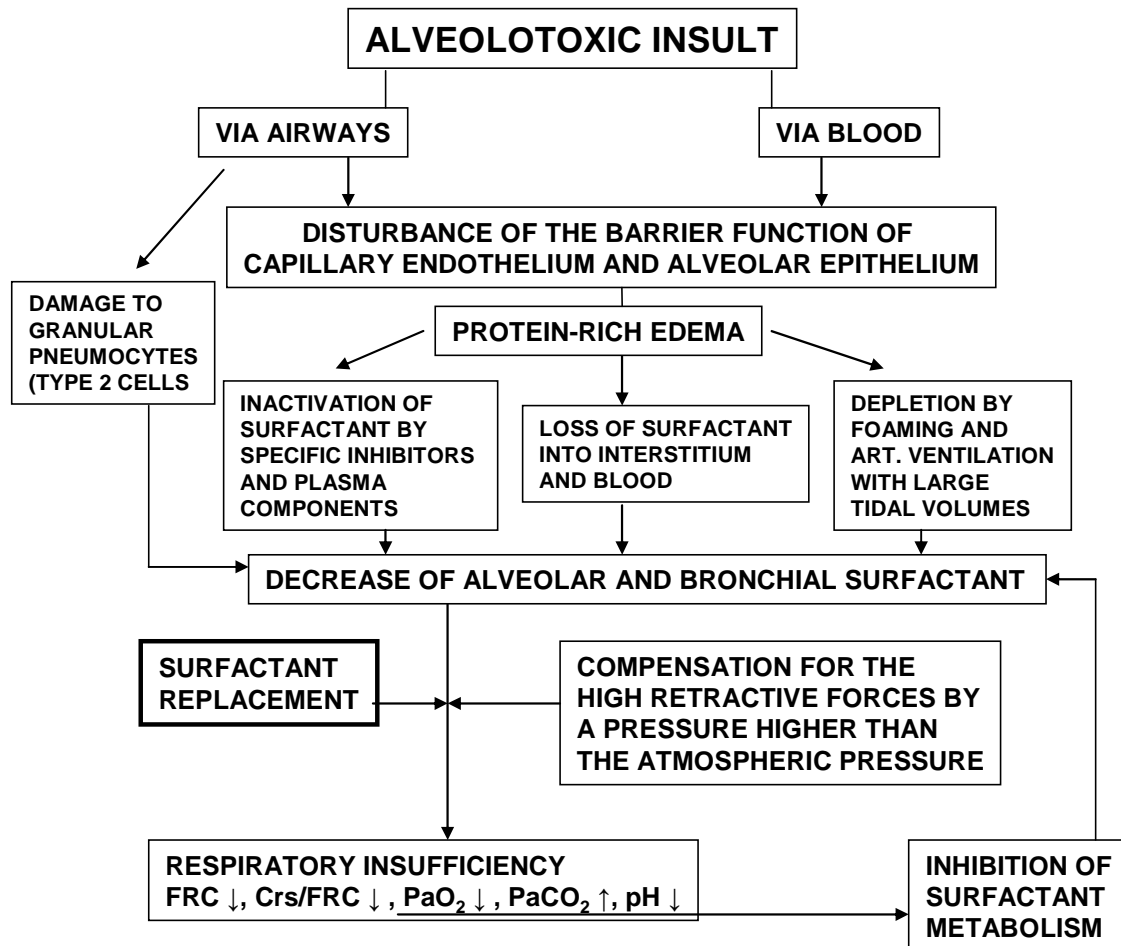


Figure 6. Pathogenesis of ARDS with special reference to the surfactant system, including suggestions to compensate for a damaged surfactant system.

Conclusion

Results from these experimental and clinical reports show that the pulmonary surfactant system is at least involved in other diseases than the neonatal RDS, such as ARDS, pneumonia, etc. We conclude that after injury to the alveolar-capillary membrane, followed by capillary leakage, the surfactant system will be responsible for further pathophysiological changes (Figure 6). These well-documented functional disturbances in the lung will finally result in the failure of the lung as a gas exchange organ.

Surfactant therapy seems a promising approach for the treatment of acute respiratory failure in ARDS and ARDS-like syndromes as well as other diseases (for an overview of

surfactant replacement therapy in clinical trials see Table 2). However, many questions remain unanswered: for example, which patients should be treated (e.g. in extended pulmonary fibrosis no effect can be expected after surfactant administration), when should surfactant treatment start, which dosage and type of surfactant should be used, the method of administration, the type of ventilatory support, and many others. To address these questions more controlled clinical trials need to be performed as soon as possible to get some answers to the questions posed.

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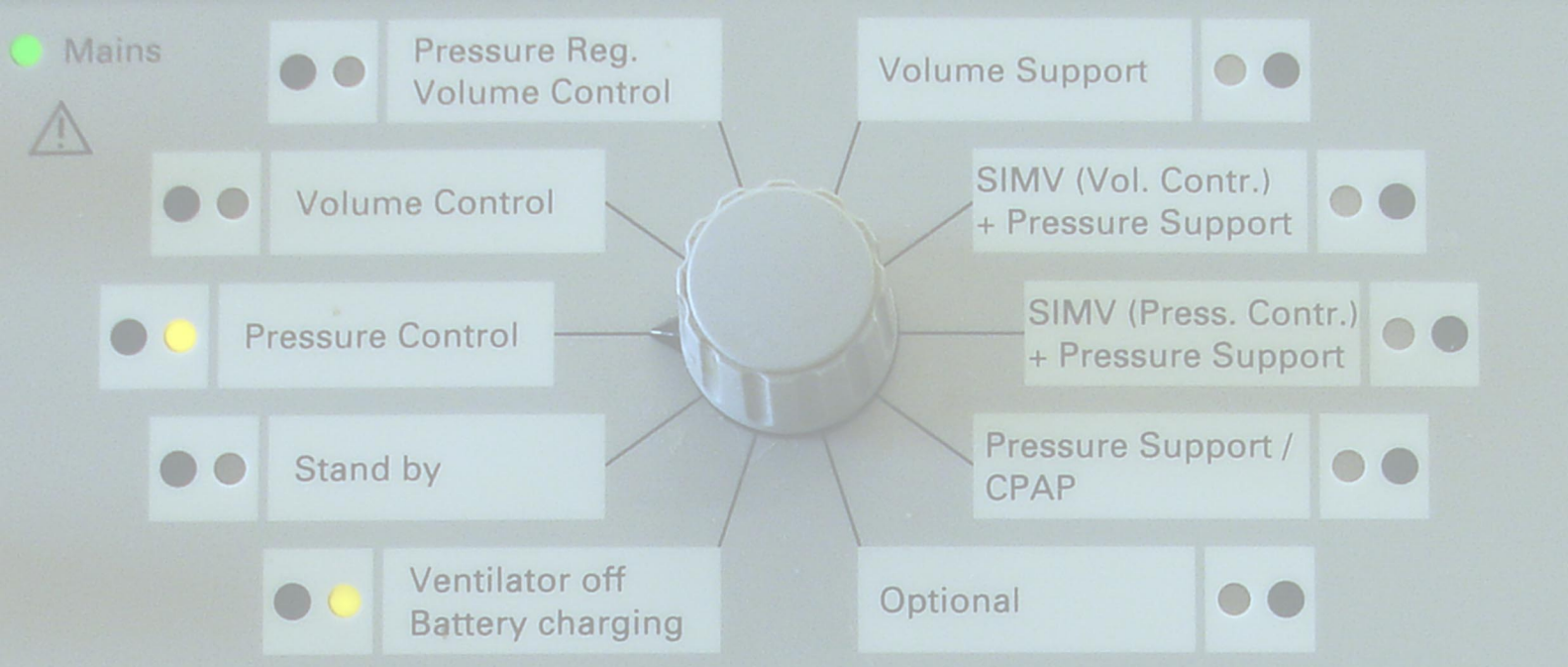
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Resp. pattern

Measured freq. b/min
50

Set freq. b/min
50

Insp. period s
0.40

Insp. flow l/s

Chapter 3

Liver-type fatty acid binding protein in serum and broncho-alveolar lavage in a model for acute respiratory distress syndrome - A possible marker for lung damage?

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Abstract

Introduction: Liver-type fatty acid binding proteins (L-FABP) have been shown to be present in alveolar macrophages and type II pneumocytes of the lung. This study determined levels of L-FABP in serum and broncho-alveolar lavage (BAL) during experimental acute respiratory distress syndrome (ARDS) to evaluate whether this molecule can serve as a marker for lung damage.

Methods: Male Sprague-Dawley rats (n=24) were ventilated and either lung lavaged or lavaged and treated with surfactant, and compared to ventilated, non-lavaged controls. Blood samples were drawn every hour for 4 h to measure L-FABP concentrations in serum. At the end of the experiment a BAL was performed to determine L-FABP levels in BAL fluid. L-FABP was measured with a sandwich ELISA.

Results: Serum L-FABP concentrations rose significantly during the first 2 h of ventilation in all groups compared to baseline values. After 2 h L-FABP levels were significantly higher in lavaged animals compared to the ventilated controls and to animals treated with surfactant. After 4 h of ventilation, L-FABP in BAL was significantly higher in lavaged, non-surfactant treated animals compared to the ventilated controls.

Conclusion: In the early phase of experimental ARDS serum L-FABP levels correlate well with the degree of lung injury.

Introduction

Acute respiratory distress syndrome (ARDS) remains one of the most severe clinical pictures on today's intensive care units, with mortality rates exceeding 40% ¹. Although many new insights into the pathogenesis of ARDS have been made during the past 20 years with related treatment approaches, only moderate progress has been made in lowering the very high mortality of ARDS. Because intervention timing is considered to be crucial, ARDS research has also focused on the search for a reliable prognostic marker ². Since the early 1980s a variety of possible markers have been described such as angiotensin converting enzyme (ACE) ³⁻⁵, leukotrienes, interleukin-8 ⁶, purines ⁷, surfactant proteins ^{8, 9} and, most recently, glycoprotein KL-6 ¹⁰. However, even though most of these markers correlated to some extent with certain disease stages of ARDS, they failed to become clinically accepted or, as with KL-6, still have to prove their value.

A potential early marker for lung injury could be the liver-type fatty acid binding protein (L-FABP), which is known to be present in alveolar macrophages ¹¹ and type II pneumocytes ¹². L-FABP belongs to the family of fatty acid binding proteins (FABPs) that were discovered in the early 1970s. The scope of functions of these proteins (weighing about 15000 daltons) is not yet fully understood. It has been shown that FABPs play a role in the modulation and regulation of gene expression and of specific enzymes within the fatty acid metabolism, as well as in the regulation and maintenance of the fatty acid fraction of cellular membranes ¹³⁻¹⁵. Also, under normal circumstances, these intracellular lipid-binding proteins are not detected except for extremely low concentrations in the blood stream ¹². Meanwhile, different FABPs have been described and are already in use as markers for cellular damage. H-FABP for instance ranks among today's established early markers for acute myocardial infarction being more sensitive for early myocardial cell injury than heart-specific creatinkinase (CK-MB) or myoglobin ¹⁶⁻¹⁸.

Inspired by the diagnostic properties of H-FABP, we investigated the effect of a lavage lung injury model on broncho-alveolar lavage (BAL) and serum levels of L-FABP to evaluate whether this molecule can serve as an early marker for lung damage.

Material and Methods

Animal preparations

The institutional Animal Investigation Committee Care approved the study protocol and handling of the animals was in accordance with the latest European Community guidelines (86/609/EC).

A total of 24 male Sprague-Dawley rats (body weight 270-350 g) were used. Anesthesia was induced with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane, Pharmachemie bv, Haarlem, the Netherlands), a sterile polyethylene catheter was inserted into a carotid artery for arterial blood sampling and a sterile metal cannula was inserted into the trachea. Anesthesia was continued with pentobarbital sodium 60 mg/kg/h bodyweight i.p. (Nembutal[®], Algin bv., Maassluis, the Netherlands). Subsequently, muscle relaxation was induced and sustained with pancuronium bromide 2 mg/kg/h bodyweight i.m. (Pavulon[®], Organon Technika, Boxtel, the Netherlands) followed by connection to the ventilator. Animals were ventilated in parallel in a pressure-controlled time-cycled mode (Servo 300, Maquet, Solna, Sweden). Initial peak inspiratory pressure (PIP) was 13 cm H₂O with a positive end-expiratory pressure (PEEP) of 3 cm H₂O, the fractional inspired oxygen concentration (FiO₂) was set at 1.0, I/E ratio of 1:2; frequency = 30 breaths per min. To re-aerate atelectatic lung areas induced by the surgical procedure, the airway pressure was increased to a PIP of 25 cm H₂O for 3 breaths. The body temperature was kept at 37°C by means of a heating pad.

Study groups

After preparation, the 24 animals were randomized into 3 groups:

Ventilation control animals (V_{control}, n=8); to test the influence of non-lung-injury factors (ventilation with 13/3 PIP/PEEP is not considered as lung injurious) such as anesthesia, intramuscular injections and surgical procedures. These animals were ventilated with a PIP of 13 cm H₂O, PEEP of 3 cm H₂O, FiO₂ of 1.0, I/E ratio of 1:2 and a frequency of 30 breaths per min throughout the course of the experiment.

Lung injury groups ARDS (n=8) and Surfactant (n=8); to induce lung injury, the lungs of these two groups were surfactant depleted by whole lung lavage (33 ml warm saline/kg bodyweight) according to Lachmann *et al.* ¹⁹ until PaO₂ was < 150 mmHg. During the lavage the ventilation pressures were set at a PIP of 26 cm H₂O and a PEEP of 6 cm H₂O. After the lavage procedure ventilator settings were set at a PIP of 28 cm H₂O and a PEEP of 8 cm H₂O and maintained for the remainder of the experiment. Ventilatory rate was set to 30 breaths per min. The animals of the Surfactant group received 5 min after the last lavage a

bolus of exogenous surfactant (150 mg/kg, HL-10, Halas Pharma GmbH, Oldenburg, Germany) intratracheally.

Measurements

Arterial blood gases (PaO₂, PaCO₂) were measured using conventional methods (ABL 555, Radiometer Copenhagen, Denmark) just before the lung lavage (baseline, b; at ventilation settings: PIP/PEEP 13/3), immediately after lavage (post lavage, pl), and at 1, 2, 3 and 4 h after lavage and mechanical ventilation (0.3 ml of blood). Blood samples for L-FABP analysis (0.7 ml) were taken at baseline and after 1, 2, 3, and 4 h; for aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) measurements at baseline and at the end of the experiment (1 ml). To replace the blood loss caused by blood sampling, all animals received 5 ml/kg Hemohees 6% (plasma expander containing hydroxyethyl starch, molecular weight 200000; Braun Melsungen, Melsungen, Germany) intra-arterially after sampling.

At the end of the experiment, all animals received an overdose of pentobarbital. Immediately after death the thorax and diaphragm was opened and pressure-volume (P/V) curves were constructed using the syringe technique, as previously described [20](#). Maximal lung compliance was calculated from the steepest slope on the deflation limb. The Gruenwald index which characterizes the surfactant system in situ [21](#), was calculated from the P/V curve, defined as $(2V_5 + V_{10})/2V_{\max}$, where V₅, V₁₀ and V_{max} are the lung volumes at airway pressures of 5, 10, and 35 cmH₂O from the deflation limb, respectively. After drawing P/V curves, all lungs were lavaged (33 ml saline/kg bodyweight) to obtain BAL fluid for L-FABP measurements.

Serum and BAL L-FABP were determined using sandwich-type enzyme-linked immunosorbent assays (ELISA). L-FABP antibodies were gained in rabbits using a rabbit-anti-FABP-antiserum (Dr. J. Pineda-Antikörper-Service, Berlin, Germany). For biotinylation, antibodies were treated with D-biotin-N-hydroxysuccinimide ester (Roche, Mannheim, Germany); these were used as detector antibodies. The detailed procedure for sandwich ELISAs can be found elsewhere [12, 22](#).

ASAT and ALAT liver enzyme analyses were performed in the Erasmus MC-Faculty clinical chemistry laboratory using standard techniques.

Intergroup differences were compared using a one-way ANOVA followed by the Tukey post hoc-test. Intragroup differences were compared using a repeated measurement ANOVA with the Turkey post hoc-test. Statistical significance was accepted at p-values < 0.05.

Results

Serum/BAL levels of L-FABP and ASAT/ALAT concentrations

Serum L-FABP was significantly increased compared to baseline values in all groups after the first two hours, with the ARDS group having the highest gain. After 2 h L-FABP levels in the ARDS group were significantly higher compared to groups Vcontrol and Surfactant, and significantly higher compared to the Vcontrol group after 3 h (Figure 1).

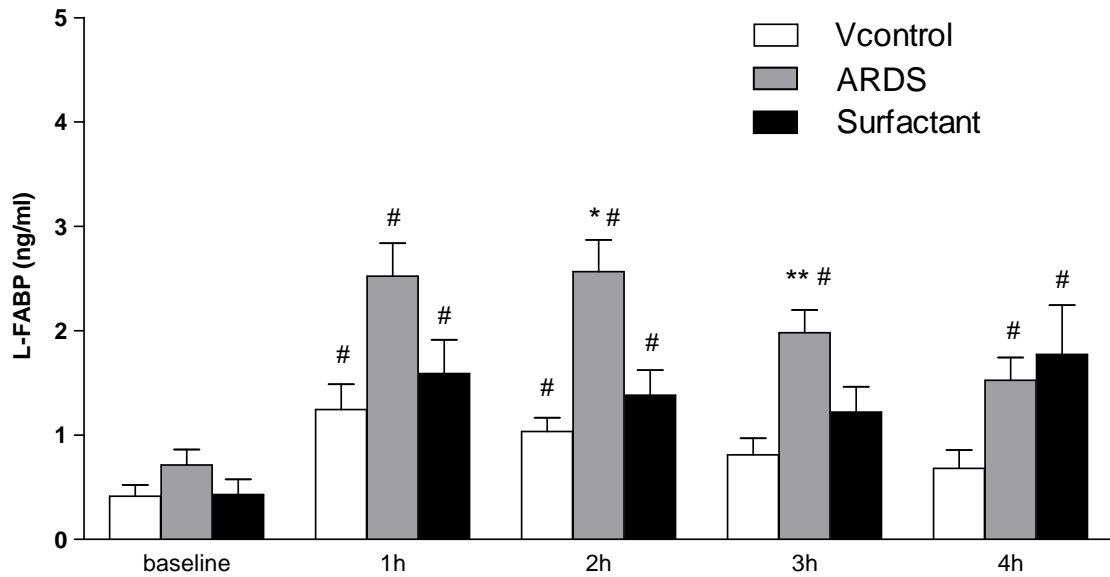


Figure 1. L-FABP serum concentration (ng/ml; mean with SEM) of the 3 groups (Vcontrol, ARDS, Surfactant) over time. b=baseline, # $p < 0.05$ vs. baseline, * $p < 0.05$ vs. all other groups at 2 h, ** $p < 0.05$ vs. Vcontrol at 3 h.

BAL L-FABP concentrations at the end of the experiment were similar in the Vcontrol and Surfactant groups, but significantly higher in the ARDS group compared to the Vcontrol group (Figure 2).

On the other hand, liver enzyme concentrations measured after 4h did not differ between the groups. There was a significant rise of ASAT after 4 h compared to baseline values in all animals, but the magnitude of this increase was not significant between the groups. Similarly, in groups ARDS and Surfactant, there were no differences in ALAT concentrations between baseline and 4 h, while ALAT levels in group Vcontrol were significantly decreased (Table 1).

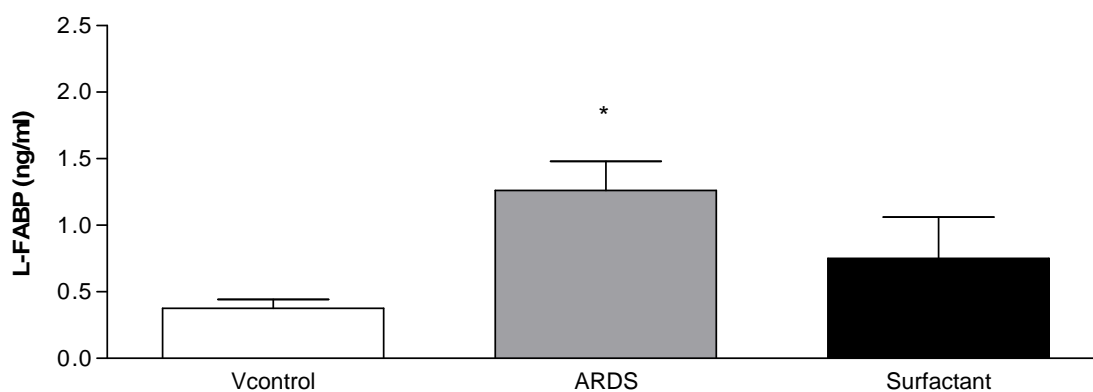


Figure 2. L-FABP concentration (ng/ml; mean with SEM) of the Vcontrol, ARDS and Surfactant groups in BAL fluid at 4 h. * $p < 0.05$ vs. Vcontrol.

Lung function

Lung lavage resulted in a significant decrease of PaO_2 in the ARDS and Surfactant groups (Table 2). Subsequent surfactant treatment led to a significant and maintained rise in PaO_2 to normal, pre-lavage levels. Following lung lavage, PaO_2 of the ARDS group remained significantly lower compared to the Vcontrol and Surfactant groups for the remainder of the experiment. As expected, PaO_2 in the Vcontrol group did not change throughout the course of the experiment and remained at healthy baseline values. There was no significant difference in PaCO_2 levels between the 3 groups (Table 2). At the end of experiment, P/V curves revealed a significantly higher maximum lung compliance (C_{max}) and Gruenwald index in the animals of the Vcontrol group compared to all others. Surfactant treatment resulted in a significantly higher C_{max} compared to the animals in the ARDS group (Table 1).

Table 1. Data on liver enzymes, maximum lung compliance (C_{max}) and the Gruenwald index of the 3 groups

	Vcontrol	ARDS	Surfactant
ASAT (U/L)	$42 \pm 37^*$	$55 \pm 36^*$	$35 \pm 41^*$
ALAT (U/L)	$-19 \pm 9^*$	1.6 ± 23	8.5 ± 16
C_{max} (ml/cm $\text{H}_2\text{O}/\text{kg}$)	5.2 ± 0.9	$2.1 \pm 0.4^\#$	$3.7 \pm 0.9^{\#\#}$
Gruenwald	$1.2 \pm 0.06^\#$	0.6 ± 0.12	1.0 ± 0.09

ASAT and ALAT given as difference between values at baseline and at 4 h. Data are mean values \pm SD, * $p < 0.05$, 4 h vs. baseline value, $^\# p < 0.05$ vs. all other groups, $^{\#\#} p < 0.05$ vs. Vcontrol

Table 2. Data on arterial blood gases of the 3 study groups

		Vcontrol	ARDS	Surfactant
PaO ₂	b	613 ± 30	596 ± 56	603 ± 27
	pl		84 ± 20	91 ± 24
	1h	580 ± 25	209 ± 130*	576 ± 127
	2h	566 ± 20	204 ± 142*	543 ± 150
	3h	551 ± 36	173 ± 134*	563 ± 159
	4h	585 ± 35	204 ± 185*	568 ± 162
PaCO ₂	b	31 ± 4	31 ± 4	33 ± 10
	pl		48 ± 12	45 ± 11
	1h	36 ± 5	45 ± 13	41 ± 5
	2h	37 ± 4	47 ± 15	39 ± 6
	3h	39 ± 5	48 ± 14	39 ± 5
	4h	39 ± 7	49 ± 17	38 ± 5

b = baseline, pl = post lavage. Data are mean values ± SD, * p<0.05 vs. all other groups

Discussion

This study shows that experimental ARDS induced by repeated lung lavage is associated with an early rise (within hours) in serum L-FABP concentration. Based on this finding it seems that L-FABP might have the potential of being a sensitive marker of early cellular lung damage.

In contrast to a previous study which found an increase of serum L-FABP in mice treated with intratracheal LPS ¹², we did not use LPS to provoke lung injury but a well established model of ARDS induced by repeated lung lavage ¹⁹. Here, surfactant is washed out leading to atelectasis formation with subsequent alveolar-capillary membrane damage due to developing shear stress ²³. Compared to using LPS, the used lavage model has the advantage of being more standardized and controllable in terms of inducing lung injury. At the same time, the PaO₂ is used as an indication of the associated degree of respiratory failure

which, on the other hand, correlates well with the degree of lung damage ¹⁹. Similar to the study of Piumngam *et al.*, we found an increase of serum L-FABP already at an early stage of the disease process, that is 2 h after lung lavage (Piumngam *et al.* made their first measurement in LPS animals after 6 h).

L-FABP is most abundant in liver cells and has already been successfully tested as a cell injury marker for liver cell damage ²⁴. Therefore, a central issue of the present study is that of the origin of the serum L-FABP, and the need to exclude that the liver acts as a possible confounder of the measured L-FABP serum levels. We identified two possible mechanisms of how our experimental setting might have induced confounding liver cell damage. First, the anesthetic used (pentobarbital) is known to be metabolized in the liver and could therefore have caused cell damage due to a toxic effect (toxic hepatitis). However, this mechanism seems unlikely since it would have caused a rise of L-FABP over time in the Vcontrol group, which was not the case (L-FABP levels in fact dropped after 1 h in this group).

The second confounding mechanism might be due to the high intrathoracic ventilation pressures possibly restricting venous return and therefore causing liver congestion. To verify that this confounder played no role in our experimental setting, we treated a second group of lavaged animals with surfactant, thereby increasing lung compliance and with it the effect of high intrathoracic pressures on decreasing venous return and possible liver congestion. The fact that the ARDS group had a significantly higher L-FABP concentration in serum 2 h after baseline compared to the surfactant group although being ventilated with the same pressures, therefore argues against this second possible confounder. In addition, the serum liver enzymes measured in all animals were either all similarly elevated compared with baseline values (ASAT) or remained unchanged (ALAT) or, in some cases, were even lower compared with baseline values (ALAT, Vcontrol).

Therefore, we conclude that the differences in serum L-FABP in the present study are indeed a function of different degrees of lung damage. This conclusion is supported by the fact that surfactant treatment attenuated lung injury, as can be seen by improved PaO₂ and lung mechanics and, consequently, also lowered serum L-FABP concentrations indicating an association between lung injury and blood L-FABP levels. Finally, the significant rise of L-FABP in the BAL fluid of the ARDS animals after 4 h further suggests that the lung can be a source of L-FABP depending on the degree of damage inflicted on lung tissue. Based on the findings of Schachtrup *et al.* and Piumngan *et al.* we postulate that the source of the measured L-FABP concentrations are alveolar macrophages and type II pneumocytes ^{11, 12}.

In conclusion, the present study provides first data of an association between different

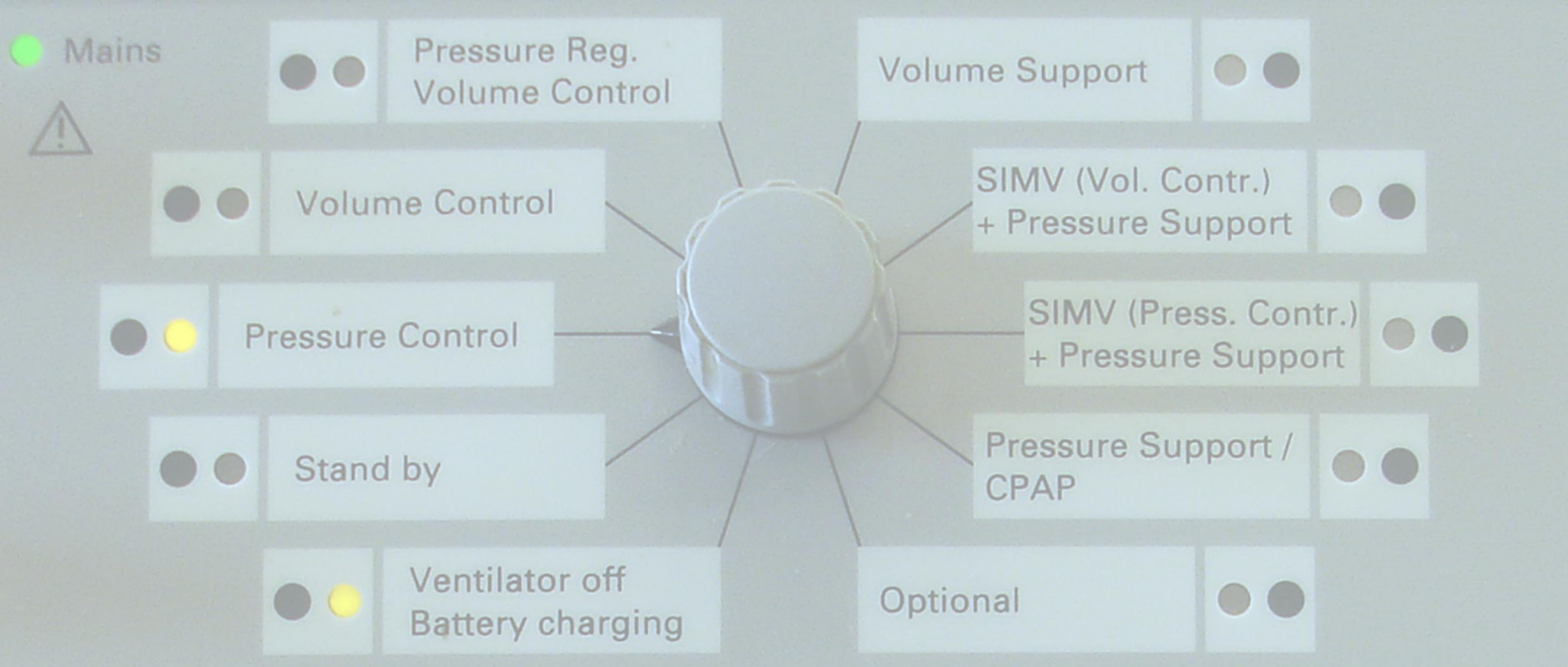
degrees of lung damage and early serum L-FABP concentrations in a standardized animal model of lung injury. Further studies are needed to evaluate a possible clinical usage of serum L-FABP as a marker of early lung injury.

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Resp. pattern

Measured freq.
b/min

50

Set freq.
b/min

50

Insp. period
s

0.40

Insp. flow
l/s

Chapter 4

Phosphoinositide 3-OH kinase inhibition prevents ventilation-induced lung cell activation

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Abstract

In acute respiratory distress syndrome (ARDS) patients, protective ventilation strategies reduce mortality and pro-inflammatory mediator levels. It has been suggested that some of the side effects of mechanical ventilation are caused by the excessive release of mediators capable of causing pulmonary inflammation and tissue destruction (biotrauma). Selective inhibition of this process might be used to minimize the side effects of artificial mechanical ventilation. The present study was designed to identify the cell types and specific signaling mechanisms that are activated by ventilation with increased pressure/volume (overventilation). In isolated perfused mouse lungs, overventilation caused nuclear translocation of nuclear factor- κ B (NF- κ B) and enhanced expression of interleukin-6 (IL-6) mRNA in alveolar macrophages (AM) and alveolar epithelial type II (ATII) cells. The phosphoinositide 3-OH kinase (PI3K) inhibitor Ly294002 prevented nuclear translocation of NF- κ B and the subsequent release of IL-6 and macrophage inflammatory protein-2 α (MIP-2 α) in overventilated, but not in endotoxic lungs. Similar results were obtained in rats *in vivo*, where Ly294002 prevented NF- κ B activation by overventilation but not by endotoxin. These findings show that AM and ATII cells contribute to the ventilation-induced release of pro-inflammatory mediators and that selective inhibition of this process is possible without inhibiting the activation of NF- κ B by endotoxin.

Introduction

Clinical studies have shown that ventilation with conventionally used high tidal volumes and pressures increases mortality, pro-inflammatory mediator release and pulmonary inflammation in acute respiratory distress syndrome (ARDS) patients ^{1, 2}. Although the correlation between mortality, ventilation and markers of inflammation in these studies does not prove a causal relationship between them, the biotrauma hypothesis is supported by an increasing number of experimental and clinical data (summarized in refs. ³⁻⁵): Stretching alveolar epithelial type II cells or alveolar macrophages in culture triggers mediator release ^{6, 7}. In isolated lungs, ventilation with increased volumes or pressures, termed overventilation ⁸, elicits local ⁹ and systemic ^{10, 11} concentrations of pro-inflammatory mediators to an extent that is comparable to that achieved by bacterial endotoxins ⁸. Protective ventilation strategies increased survival and reduced mediator release in animal models of acute lung injury, e.g. after lung lavage ¹²⁻¹⁴ or acid instillation ¹⁵⁻¹⁷. It was also demonstrated that the chemokine macrophage inflammatory protein-2 α (MIP-2 α) and its receptor (CXCR2) are critical mediators of ventilation-induced lung injury ¹⁸. In ARDS patients, Ranieri and colleagues noted a close correlation between ventilation-associated release of IL-6 and multi-organ failure ¹⁹. And most recently, it was shown that ventilation of patients with increased pressures leads to an elevation in pro-inflammatory mediators including IL-6 that was reversible if the ventilation pressures were reduced ²⁰. This last finding establishes a causal link between ventilation and rapid cytokine release in patients.

Key questions in this area concern the cell types and the signaling cascades that become activated by ventilation. Experiments with stretched alveolar macrophages ⁶, intact mouse lungs ⁸ and healthy rat lungs ²¹ have led to the conclusion that a central event in the signaling cascade elicited by overventilation is activation, i.e. nuclear translocation of the transcription factor nuclear factor- κ B (NF- κ B). Overventilation activated NF- κ B also in lipopolisaccharide (LPS)-resistant toll-like receptor (TLR4)-deficient mice, demonstrating that different signaling pathways lead to activation of NF- κ B by ventilation and by LPS ⁸. As pointed out ²², this suggests that it may be possible to selectively target the pro-inflammatory side effects of mechanical ventilation, without interfering with the organisms' ability to respond to bacterial infections.

Phosphoinositide 3-OH kinases (PI3K) are a family of ubiquitous heterodimeric lipid-modifying enzymes divided into three classes ²³. PI3K play an important role in mitogenic signaling and cell survival, cytoskeletal remodeling, metabolic control and vesicular

trafficking [23](#). Several *in vitro* studies have established a link between PI3K and activation of NF- κ B. These studies can be divided into those supporting the sequence PI3K \rightarrow Akt (protein kinase B) \rightarrow NF- κ B [24-30](#), and those that show activation of NF- κ B by PI3K by pathways that apparently are independent of Akt [31-33](#).

Here, we investigated the hypothesis that PI3K contributes to the activation of NF- κ B and release of IL-6 and MIP-2 α during ventilation with high ventilation pressures (over-ventilation), but not LPS exposure. We focused on IL-6 and MIP-2 α , because both mediators are released in great quantities by overventilation [8](#) and have been shown to be relevant in clinical [1, 19, 20](#) respectively experimental [18](#) conditions of ventilator-associated lung injury. In addition, we used immunohistochemistry and *in situ* hybridization to identify the cell types in which NF- κ B and IL-6 become activated by overventilation. We should emphasize that the present study was not designed to directly study acute lung injury, but to characterize the signaling pathways that lead to inflammatory gene activation during ventilation. While we did not study lung injury itself, there is clear evidence that pro-inflammatory mediators such as MIP-2 α play an essential role in biotrauma [18](#). Hence, understanding the molecular mechanisms of ventilation-induced cell activation and mediator release will help to develop strategies to minimize the side effects of mechanical ventilation.

Materials and methods

Mice

Female BALB/C mice were obtained from the breeding house of the Research Center Borstel. All animals were used at a weight of 20-23 g.

Materials

Pentobarbital sodium (Narcoren[®]) was purchased from Merial GmbH (Hallbergmoos, Germany); RPMI 1640 was from BioWhittaker (Verviers, Belgium); the PI-3-Kinase inhibitor Ly294002 from Sigma-Aldrich (Taufkirchen, Germany).

Isolated perfused mouse lung preparation (IPL)

The mouse lungs were prepared and perfused essentially as recently described [8](#), [11](#), [34](#). Briefly, lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1 ml/min resulting in a pulmonary artery pressure of 2 to 3 cm H₂O. As a perfusion medium we used RPMI medium lacking phenol red (37°C) and containing 4% low endotoxin grade albumin. Under control conditions, the lungs were ventilated with room air by negative pressure (-3 cm H₂O to -10 cm H₂O) at a rate of 90 breaths/min resulting in a tidal volume (V_T) of about 200 µl. Artificial thorax chamber pressure was measured with a differential pressure transducer (DP 45-24; Validyne, Northridge, CA), and the airflow rate was measured with a Fleisch-type pneumotachograph tube connected to a differential pressure transducer (DP 45-15, Validyne). Arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne, Irvine, CA) that was connected to the cannula ending in the pulmonary artery. All data were transmitted to a computer and analyzed with Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). pO₂ and pCO₂ levels were measured in the effluent perfusate with an IL 1620 blood gas analyzer (Instrumentation Laboratories, Kirchheim, Germany).

Experimental design

In perfused mouse lungs, overventilation with negative or positive pressure leads to the same extent of mediator release [11](#). From this observation we concluded that it is the transpulmonary

pressure and not the change in vascular pressure that is responsible for the overventilation-induced mediator release. In the present study we used negative pressure ventilation, because the low perfusion pressures that result from negative pressure ventilation reduce vascular shear stress³⁵ and help to minimize hydrostatic edema formation.

Ventilation was always pressure-controlled. In all experiments, the lungs were first perfused and ventilated for 60 min under baseline conditions with an end-inspiratory pressure (EIP) of -10 cm H₂O and an end-expiratory pressure (EEP) of -3 cm H₂O resulting in tidal volumes of about 200 μ l (Fig. 1). Subsequently, the lungs were randomly allocated to one of the following five groups and were perfused and ventilated for another 180 min: group 1 with a low EIP of -10 cm H₂O (control); group 2 with a high distending pressure of -25 cm H₂O (OV); group 3 was OV (as in group 2) and pre-treatment with 50 μ M Ly294002 dissolved in 0.05% DMSO (OV/Ly) from 30 min before OV on; group 4 with a low EIP of -10 cm H₂O and 50 μ g/ml LPS (LPS); group 5 was LPS (as in group 4) and pre-treatment with 50 μ M Ly294002 dissolved in 0.05% DMSO (LPS/Ly) from 30 min before OV on. Some of the experiments were interrupted after 60 min of OV- or LPS-treatment for electromobility shift assay (EMSA), Western Blots, immunohistochemistry or *in situ* hybridization.

In vivo study in rats

Animals were anesthetized with 65% NO₂/ 35% O₂ and 2% isoflurane and a sterile metal cannula was inserted into the trachea as described before²¹. After these surgical procedures, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg pentobarbital sodium i.p. (Nembutal; Algin, Maassluis, The Netherlands). Subsequently, muscle relaxation was induced by pancuronium bromide 2 mg/kg, i.m. (Pavulon; Organon Technika, Boxtel, The Netherlands), and ventilation was initiated with a Servo Ventilator 300 (Siemens Elema, Solna, Sweden) in a pressure-constant time-cycled mode and at a FiO₂ of 1.0. Rats were mechanically ventilated with normal (13/3 PIP (positive inspiratory pressure)/PEEP (positive end expiratory pressure) in cm H₂O) or with high pressure (45/10) for 30 min, leading to tidal volumes of approximately 12 ml/kg and 18 ml/kg³⁶, respectively. At the end of the study period, the animals were killed with an overdose of pentobarbital, the thorax was opened and lungs were collected sterile, snap frozen and stored at -80°C.

The animals were randomly allocated into 5 experimental groups of 6 animals each: (1) non-ventilated untreated controls; (2) injection of the solvent (0.4 mg/kg DMSO, i.v.) 10 min before ventilation with 45/10 cm H₂O for 30 min; (3) injection of Ly294002 (1.4 mg/kg,

i.v.) 10 min before ventilation with 45/10 cm H₂O for 30 min ventilation; (4) injection of the solvent (0.4 mg/kg DMSO, i.v.) 10 min before injection of LPS (8 mg/kg) and ventilation with 13/3 cm H₂O for 30 min; (5) injection of Ly294002 (1.4 mg/kg, i.v.) 10 min before injection of LPS (8 mg/kg) and ventilation with 13/3 cm H₂O for 30 min.

Analysis of NF- κ B by EMSA

EMSA was performed as described before ⁸. For the antibody supershifts, a NF- κ B electrophoretic mobility-shift assay kit was purchased from Geneka Biotechnology Inc. (Montreal, Canada) and performed according to the suppliers instructions. In brief, the ready-to-label-NF- κ B probe was end-labeled with ³²P- γ -ATP (Hartmann, Braunschweig, Germany), using T4 kinase (Roche, Mannheim, Germany), and unincorporated nucleotides were removed with a G-25 Sephadex separation column (Amersham Pharmacia Biotech Inc., Freiburg, Germany). Nuclear extracts prepared from the lungs were pre-incubated with the appropriate buffers at 4°C for 20 min. Subsequently, the probe premix (\approx 30,000 cpm) was added and incubated for another 20 min at 4°C. The DNA-protein complexes were resolved in a native 4% polyacrylamide gel in 0.5 x Tris borate-EDTA buffer. For the antibody supershift analysis, antibody specific to the p50 or p65 NF- κ B subunit was added to the extract premix containing the nuclear extract. The resulting complex was analyzed in the same 4% polyacrylamide gel.

Western blot analysis

Western Blot analysis was performed as described before ²¹. Frozen lungs were powdered with a pestle in the constant presence of liquid nitrogen. Aliquots of the lung powder were lysed and homogenized in a buffer (50 mM Tris-Cl, pH 6.8, 150 mM NaCl, and 1% Triton X-100) containing Pefablock (1mM), aprotinin (1 μ g/ml), pepstatin (1 μ g/ml), leupeptin (1 μ g/ml), NaF (1mM), Na₃VO₄ (1mM), β -Glycerolphosphate (1mM). After 20 min on ice, the lysates were collected by pelleting the cellular debris for 15 min at 16,000 x g. Total protein content was determined by a commercially available test (PIERCE, Rockford, IL, USA).

An equal amount of protein (60 μ g/slot) was size-fractionated by standard SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Dassel, Germany) and then immunoblotted with primary antibodies

(New England Biolabs (NEB), Frankfurt, Germany) and horseradish peroxidase-conjugated secondary antibodies (NEB). Detection of the bound antibody with LumiGLO chemiluminescent substrate was performed according to the manufacturer (NEB). The densitometric analysis was performed with OPTIMAS 6.2 software (Optimas Corporation, Bothel, WA, USA).

IL-6 and MIP-2 α measurements

Perfusate concentrations of IL-6 and MIP-2 α were determined by ELISA (R&D Systems GmbH, Wiesbaden, Germany) at the time points indicated in Fig. 10. The detection limit for IL-6 and MIP-2 α was 10 pg/ml and 5 pg/ml, respectively.

Immunohistochemistry

NF- κ B and I κ B- β . For immunohistochemistry, the perfused lungs were instilled *via* the trachea with 4% paraformaldehyde in PBS at an airway pressure of 20 cm H₂O. The trachea was tightly ligated, and the lungs stored in the same fixative in the cold. After removal of the trachea, the lungs were embedded into melted 2% agarose in PBS at 38-40°C. After hardening of the agarose at 4°C, the lungs were cut into equidistant slices of about 2 mm in thickness, which were then embedded into paraffin. Dewaxed paraffin sections were stained by means of established immunohistochemical procedures described in detail in previous studies [38](#), [39](#). In brief, sections were subjected to antigen retrieval using microwave treatment (2 times 5 min at 750W) in citrate buffer (pH 6.0). Then, the sections were incubated with hydrogen peroxide to eliminate endogenous peroxidase, and unspecific staining was blocked using 50% fetal calf serum in PBS prior to incubation with the primary antibodies. To detect nuclear translocation of NF- κ B p65, a monoclonal mouse antibody was used (MAB3026; Chemicon International, Temecula, CA), which recognizes an epitope overlapping the nuclear location signal (NLS) of the p65 subunit of the NF- κ B heterodimer. This antibody has been successfully used to detect NF- κ B translocation in previous studies (e.g. 40). Additionally, an affinity purified polyclonal goat antiserum mapping within the amino terminal domain of NF- κ B p65 was used (sc-109; Santa Cruz Biotechnology, Santa Cruz, CA), with which identical results were obtained. Degradation of the cytoplasmic NF- κ B inhibitor I κ B- β was studied using an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of I κ B- β of

mouse origin (Santa Cruz Biotechnology, CA), which has previously been reported to selectively detect I κ B- β in paraffin embedded mouse and rat tissues ⁴¹. After washing in PBS for 10 min, the monoclonal mouse antibody against NF- κ B p65 was detected by the avidin-biotin peroxidase complex (ABC) technique using a mouse-on-mouse kit according to the manufacturer's recommendations (Vectastain Elite kit, Vector Laboratories, Burlingame). The polyclonal rabbit antiserum against I κ B- β was detected by incubation for 60 min with peroxidase-coupled goat anti-rabbit IgG (HRP77; Dr. Grossmann, Dresden) at a 1:400 dilution in PBS containing 50% fetal calf serum followed by diaminobenzidine for 8 min. After rinsing in PBS, brief counterstaining with hemalum was performed. The sections were coded, and then examined in a blinded fashion with an Olympus BH-2 microscope (Olympus, Tokyo, Japan).

In situ Hybridization

HOPE-fixed, paraffin-embedded specimens were prepared as previously described ⁴². Total RNA was extracted from lung specimens according to the manufacturer's recommendations (RNeasy, Qiagen, Hilden, Germany). After destroying residual DNA by treatment with DNase (Gibco, Karlsruhe, Germany), cDNA was synthesized by reverse transcription (Superscript, Gibco) using 1 μ g of total RNA and an oligo dT15 primer as previously described ^{43, 44}. PCR was done by targeting a 649 bp fragment of murine IL-6 (IL-6 forward: ACC GCT ATG AAG TTC CTC TC, IL-6 reverse: ATA ACG CAC TAG GTT TGC CG). For amplification 5 μ l cDNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP (Promega), 5 units Taq-Polymerase (Gibco) and 0.8 μ M of each primer have been used in a final volume of 50 μ l. 40 cycles of amplification followed, each consisting of 94°C 1 min, 57°C 1 min, 72°C 1.5 min. The final extension of the products was performed by incubation at 72°C for 15 min. PCR-products were separated on 2% agarose gels and stained with ethidiumbromide. Probes were labeled with digoxigenin by random primed labeling using High-Prime (Roche) overnight according to the manufacturer's recommendations as described ⁴³. Concentrations of the labeled probes were estimated by comparison with control DNA of a given concentration and direct detection of the probes spotted onto positively-charged nylon membranes ⁴³.

The hybridization solution was composed of 3 ng/ μ l freshly denatured probe, 0.1% SDS, 50% formamide and 250 μ g/ml yeast tRNA (Roche). Hybridization was carried out overnight in moist chambers at 48°C. Slides were washed as follows: 2 x SSC twice for 10 min at ambient temperature, then 0.2 x SSC twice for 30 min at 50°C. Detection: Slides were washed in DIG washing buffer for 1 min at ambient temperature. Blocking reagent (1%) in maleic acid buffer (Roche) was then applied for 30 min at ambient temperature. An anti-digoxigenin antibody conjugated with alkaline phosphatase (anti-DIG-AP, Roche) was applied in a dilution of 1:7500 in 1% blocking reagent for 30 min at ambient temperature. Slides were washed in washing buffer twice, allowing 15 min for each washing step. To achieve optimal pH for alkaline phosphatase color reaction, slides were incubated in detection buffer (Roche) at a pH of 9.5 for 5 min at ambient temperature.

We used new-fuchsin as a chromogen for alkaline phosphatase. The color substrate solution was applied at ambient temperature; the staining process was observed under a microscope and stopped after 10 min by incubation in demineralized water. Counterstain was performed with Mayer's hemalum for new-fuchsin and AEC or nuclear fast red for NBT/BCIP. Slides were mounted with Kayser's glycerine gelatine. Omission of the probe or the detection antibody as well as RNase treatment of the sections served as negative controls.

Statistical analysis

The data are shown as mean \pm SEM. The time courses of the IL-6 and MIP-2 α data in Fig. 10 were analyzed by Repeated Measurement ANOVA (from 60 min to 240 min) and orthogonal polynomials (JMP 4.05, SAS Institute, Cary, N.C., USA). The linear trend was highly significant ($P < 0.0051$), and the subsequent contrasts were corrected by the Shaffer procedure ⁴⁵. The densitometric data from the EMSA were analyzed by the Kruskal-Wallis test followed by the one-tailed Mann-Whitney test (SPSS 11.5, SPSS Software GmbH, München, Germany); multiple comparisons were again adjusted by the Shaffer procedure.

Results

Tidal volume

Initially, all lungs were perfused for 60 min with 1 ml/min and ventilated with -10/-3 cm H₂O end-inspiratory pressure / end-expiratory pressure (EIP/EEP) (control conditions). To exclude binding of Ly294002 to proteins in the perfusate, subsequently all lungs were perfused with medium devoid of albumin. The lack of serum did not affect tidal volume (Fig. 1), pulmonary compliance, vascular resistance or histological appearance of the lungs (data not shown). After 60 min, either control ventilation was continued or overventilation (-25/-3 cm H₂O EIP/EEP) was started. Overventilation more than doubled tidal volume and this response was not affected by the PI3K-inhibitor Ly294002 (Fig. 1). The tidal volume of lungs perfused with LPS or with Ly294002/LPS was not different from controls (data not shown) as shown before ⁸. These findings suggest that Ly294002 was well tolerated and had no adverse effects on lung physiology. In line with this, the pO₂ or pCO₂ levels of the perfusate medium were not different whether animals were overventilated in the presence or absence of Ly294002 (Fig. 2).

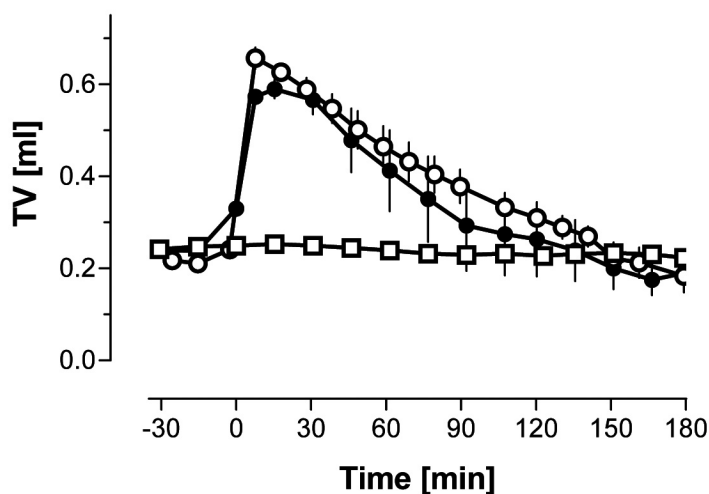


Figure 1. Tidal volume of isolated perfused lungs ventilated with an end-inspiratory pressure (EIP) of either -10 cm H₂O (squares, n=3) or -25 cm H₂O (circles, n=7). Filled circles indicate lungs that were ventilated with -25 cm H₂O EIP and treated with 50 μM Ly294002 (n=4). Data are mean ± SEM.

Kinase activation

The serine kinase Akt is known to be activated by PI3K, so phosphorylation of Akt may serve as an indirect evidence for activation of PI3K. Overventilation caused activation of Akt in the lung homogenate, while the effect of LPS was much weaker (Fig. 3). The PI3K inhibitor Ly294002 abolished the effect of overventilation on Akt (Fig. 3). To exclude an effect of Ly294002 on other kinases such as MAPK, we investigated the effects of Ly294002 on phosphorylation of the MAP kinases Erk 1/2 and p38 (Fig. 3). Overventilation for 60 min increased phosphorylation of Erk 1/2, which was only slightly affected by Ly294002

(compared to DMSO control). In contrast, the MEK-inhibitor U0126 reduced Erk 1/2-phosphorylation below baseline levels. Overventilation for 60 min had no effect on phosphorylation of p38-kinase. Neither Ly294002 nor U0126 affected the basal phosphorylation of p38.

NF- κ B

We next investigated whether PI3K contributes to activation of NF- κ B. Pre-treatment with Ly294002 prevented the nuclear translocation of NF- κ B induced by OV, but not by LPS (Fig. 4). To establish the physiological relevance of these findings, we examined the effect of Ly294002 on activation of NF- κ B in rats *in vivo*. Both overventilation and LPS induced NF- κ B in rats *in vivo* (Fig. 5), although here LPS was clearly more potent as indicated by the fact that in order to get a reliable signal in the EMSA we had to load the gels with 40 μ g protein in the case of OV, but only 10 μ g in the case of LPS. Intravenous injection of Ly294002 largely prevented NF- κ B activation induced by overventilation, but not by endotoxin (Fig. 5.)

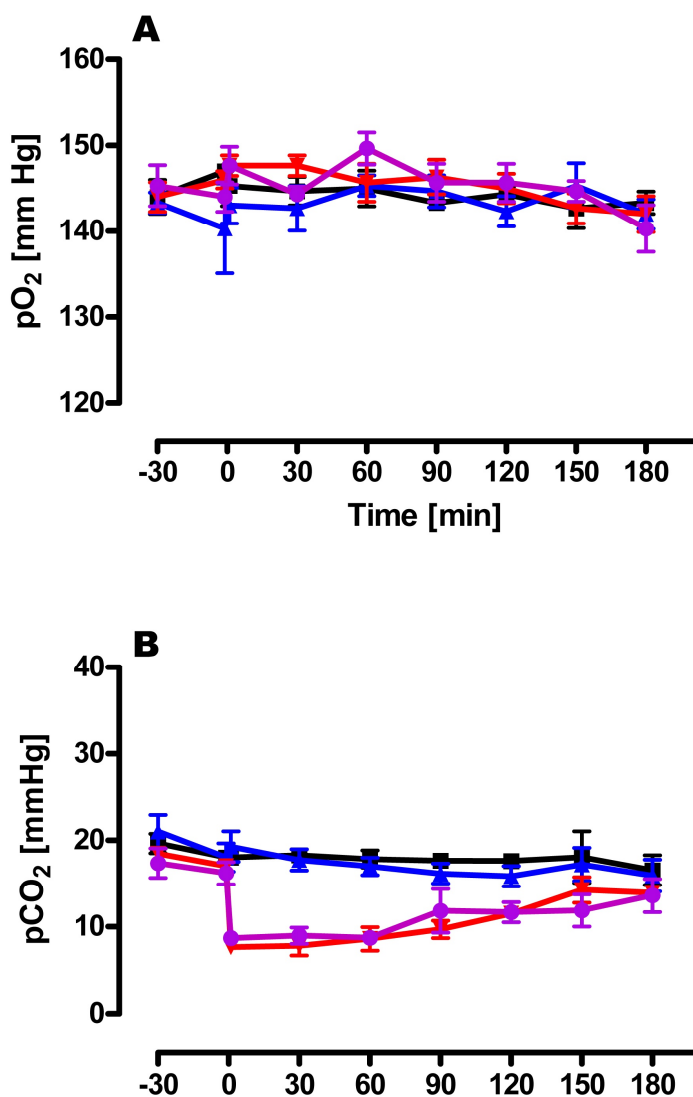


Figure 2. Blood gases in the effluent buffer. Perfusate samples were taken every 30 min and analyzed immediately. All lungs were ventilated with -3/-10 cm H₂O EEP/EIP for the first 30 min. At time point 0, control lungs (black squares) and LPS-treated lungs (50 μ g/ml, blue triangles) were further on ventilated with -3/-10 cm H₂O EEP/EIP; overventilated lungs with (50 μ M, pink circles) or without (red inverted triangles) LY294002 were ventilated with -3/-25 cm H₂O EEP/EIP. Data are mean \pm SEM, n=3. Please note that unlike blood that enters the pulmonary artery the perfusion buffer was not deoxygenated, thus all pO₂ values are in the range of the solubility of oxygen in water at 37°C and do not change between groups.

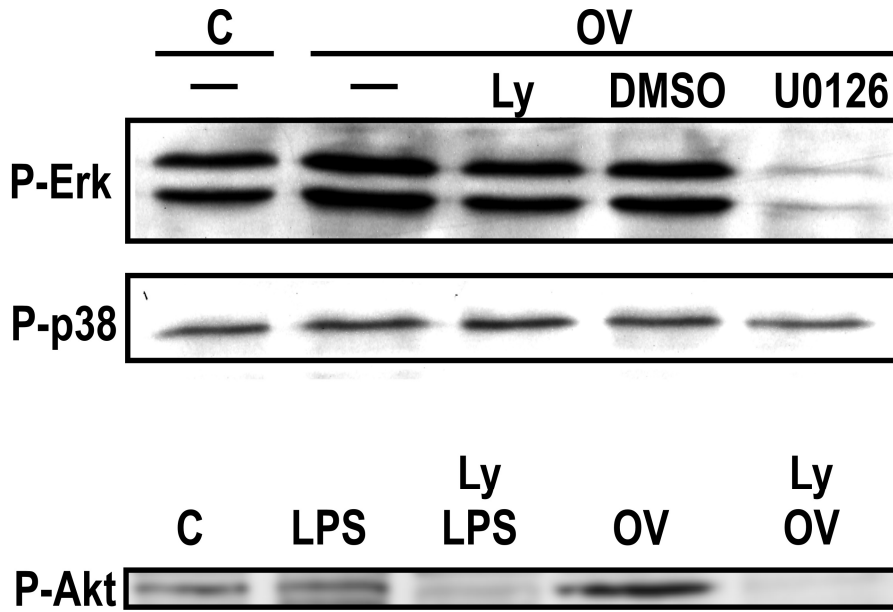


Figure 3. Effect of Ly294002 (Ly) and U0126 on phosphorylation of Erk 1/2, p38 and Akt in isolated perfused mouse lungs. P-Erk (a), P-p38 (a) and P-Akt (b) were analyzed by immunoblot, using antibodies specific for the phosphorylated forms. Shown are data from lungs ventilated for 60 min with -10/-3 cm H₂O EIP/EEP (C), -25/-3 cm H₂O EIP/EEP (OV) or 50 µg/ml LPS in the presence of 50 µM Ly294002 (Ly), 0.05% DMSO or 20 µM U0126. Similar results were

obtained in three independent experiments. DMSO alone had no effect on the phosphorylation of Akt (data not shown).

To identify the cell type in which OV activates NF-κB, we used indirect immunohistochemistry with antibodies against p65 and I-κBβ. These antibodies were selected, because overventilation elicited nuclear translocation of the p65 (also p50) NF-κB subunit (Fig. 6), and because PI3K activity is known to lead only to a transient decrease in I-κBα, but a sustained one in I-κBβ ⁴⁶.

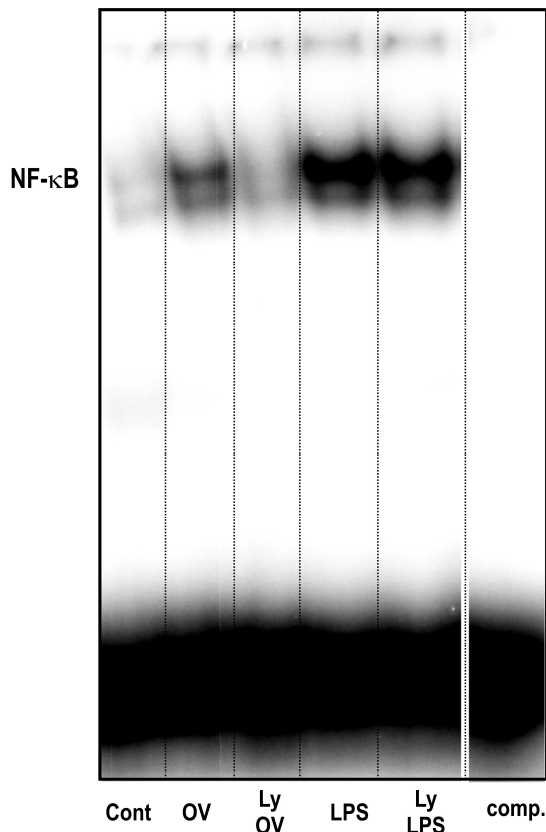


Figure 4: Differential inhibition of nuclear translocation of NF-κB by inhibition of PI3 kinase in mouse lungs. Lungs were ventilated under control conditions for 60 min, before they were ventilated with -10/-3 cm H₂O EIP/EEP in the absence (Cont) or presence (LPS) of 50 µg/ml LPS or with -25/-3 cm H₂O EIP/EEP (OV) for another 60 min. Some of the OV- or LPS-treated lungs were treated with 50 µM Ly294002 (Ly). Nuclear NF-κB translocation was determined by EMSA; each gel slot was loaded with 10 µg protein. The rightmost band (comp) shows competition of band 4 (LPS) in the presence of unlabeled oligonucleotides. Similar results were obtained in three independent experiments.

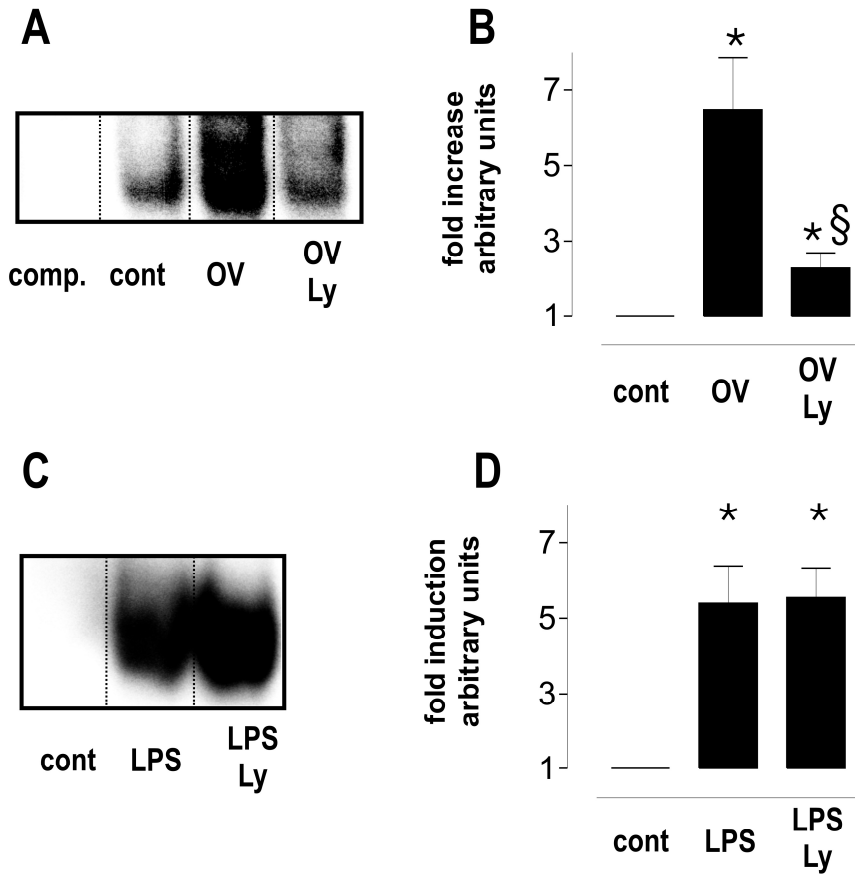


Figure 5.

Differential inhibition of nuclear translocation of NF-κB by inhibition of PI3 kinase in rats *in vivo*. Panel A and C show representative EMSA images, panel B and D show the densitometric quantification of these EMSAs. Panel A and B show data from rats ventilated for 30 min with 45/10 cm H₂O (OV) with and without pretreatment with 1.4 mg/kg Ly294002. Each gel slot was loaded with 40 μg protein. Panel C and D show data from rats ventilated for 30 min with 13/3 cm H₂O and treated with 8 mg/kg LPS with and without pretreatment with 1.4 mg/kg Ly294002. Each gel slot was loaded with 10 μg protein. Comp,

cold competition of band OV in panel A; cont, unventilated untreated controls. Data are mean ± SEM from 5 or 6 animals per group. *, p < 0.05 vs. cont; §, p < 0.05 vs. OV.

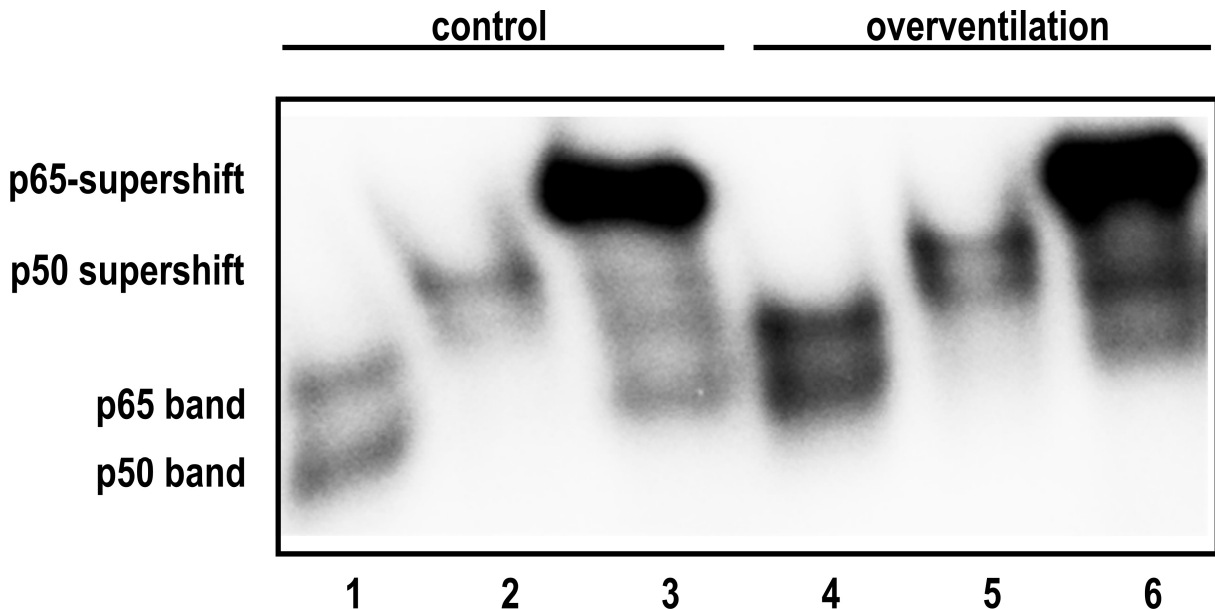


Figure 6. Overventilation increases DNA binding of p65 and p50 subunits of NF-κB isolated perfused mouse lungs. Nuclear extracts from lungs ventilated for 60 min with -10/-3 cm H₂O EIP/EEP (control, lane 1) or -25/-3 cm H₂O EIP/EEP (overventilation, lane 4) were analyzed by EMSA. For supershift assays, antibodies specific to p50 (lane 2 and 5) and p65 (lanes 3 and 6) subunits of NF-κB were added to the DNA binding mixture. Specificity of these DNA protein bands was verified by DNA competition as shown in Fig. 5. Similar results were obtained in three independent experiments.

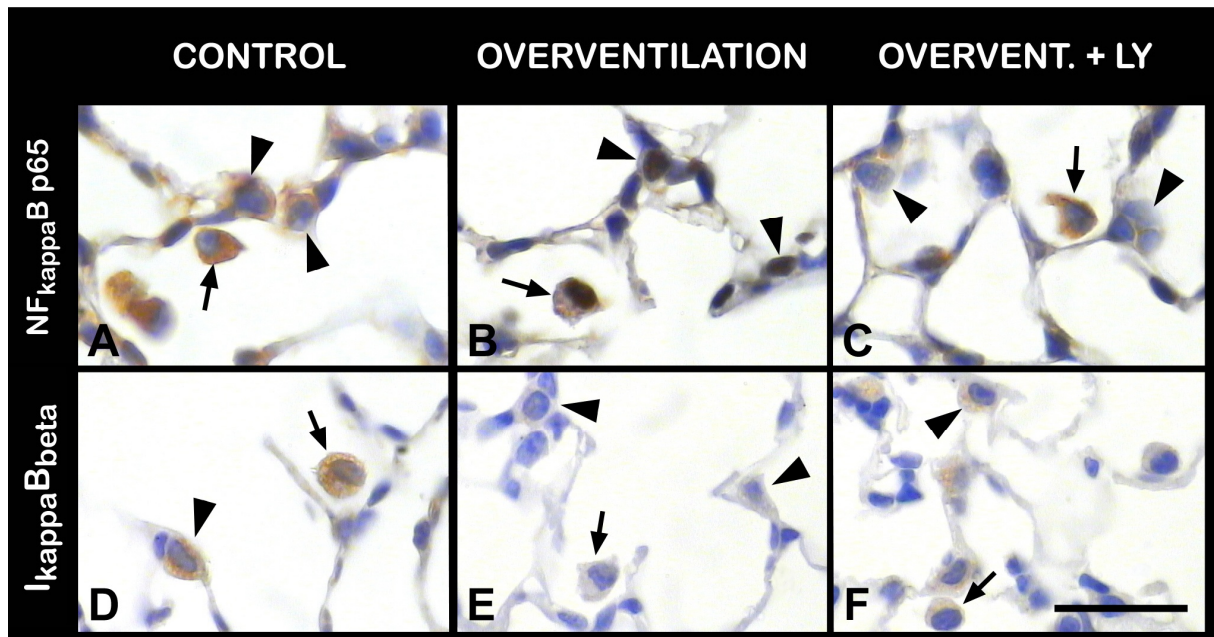


Figure 7. Indirect immunohistochemical localization of NF- κ B p65 (A-C) and the NF- κ B inhibitor I- κ B β (D-F) in isolated perfused mouse lungs. Shown are high-power micrographs from lungs that were ventilated with either -3/-10 cm H₂O EEP/EIP (control, panels A and D) or with -3/-25 cm H₂O EEP/EIP (overventilation, panels B and E) and overventilated lungs, which were treated with the PI3-kinase inhibitor Ly294002 (panels C and F). Cell types that after overventilation exhibited translocation of NF- κ B p65 into the nucleus (B) and loss of cytoplasmic I- κ B β (E) were identified as alveolar macrophages (arrows) and alveolar epithelial type II cells (arrowheads). The micrographs are representative of three independent experiments each. Bar = 25 μ m.

In control lungs, immunoreactivity for NF- κ B p65 was almost completely restricted to the cytoplasm of parenchymal cells (Fig. 7A, Fig. 8). In all groups, nuclear localization of NF- κ B p65 was regularly seen in endothelial cells of small venules only, and in very few bronchiolar epithelial cells (data not shown). In contrast, overventilation resulted in a marked increase in parenchymal cells exhibiting nuclear localization of NF- κ B p65 (Fig. 7B; Fig. 8 panels B and E). Translocation of NF- κ B p65 was mainly but not exclusively seen in alveolar macrophages and alveolar epithelial type II cells. Nuclear localization of NF- κ B p65 was clearly decreased, albeit not to control levels, in overventilated lungs treated with the PI3K inhibitor Ly294002 (Fig. 7C; Fig. 8 panels C and F).

Correspondingly, immunoreactivity for the NF- κ B inhibitor I- κ B β was exclusively seen in the cytoplasm of bronchiolar epithelial cells, alveolar macrophages, and alveolar epithelial cells type II in control lungs. With the exception of bronchiolar epithelial cells, immunoreactivity for I- κ B β was completely lost in parenchymal cells after overventilation, which was in part prevented by Ly294002 (Fig. 7 D-F; Fig. 8 panels G-I).

IL-6 expression

To demonstrate that the activation of NF- κ B in alveolar macrophages and alveolar epithelial cells type II corresponded to increased gene expression, we analyzed the expression of IL-6 mRNA in these cells (Fig. 9). After 60 min of overventilation, IL-6 mRNA was increased in alveolar macrophages and alveolar epithelial type II cells, and again, this response was prevented by Ly294002.

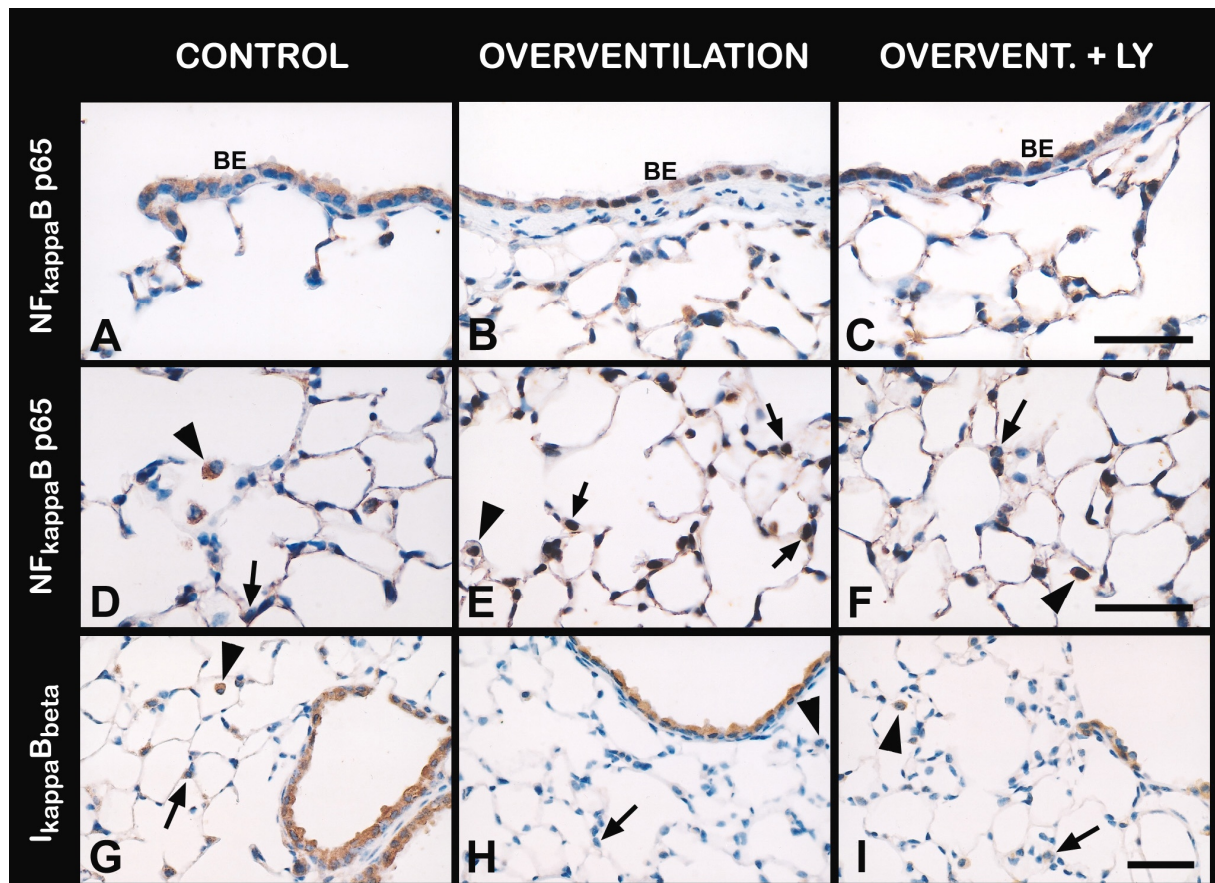


Figure 8. Indirect immunohistochemical localization of NF- κ B p65 and the NF- κ B inhibitor I κ B- β in isolated perfused mouse lungs. Shown are high-power micrographs from lungs that were ventilated with either -3/-10 cm H₂O EEP/EIP (control) or -3/-25 cm H₂O EEP/EIP (overventilation) and overventilated lungs, which were treated with the PI3-kinase inhibitor Ly294002. The upper row (A-C) demonstrates localization of NF- κ B p65 to the bronchiolar epithelium (BE) with nuclear localization seen in only a few epithelial cells (B). The middle row (D-F) demonstrates a marked increase in parenchymal cells exhibiting nuclear localization of NF- κ B p65 in overventilated lungs (E) in comparison with control lungs (D) and Ly294002 treated lungs (F). Translocation of NF- κ B p65 was seen in alveolar macrophages (arrowheads), alveolar epithelial type II cells (arrows), which are located at alveolar corners, as well as in some other unidentified cells of the alveolar septum. The lower row (G-I) displays the corresponding pattern in the immunoreactivity for the NF- κ B inhibitor I κ B- β . Cytoplasmic I κ B- β seen in control lungs was completely lost in parenchymal cells after overventilation (H), which was in part prevented by Ly294002 (I). In contrast, bronchiolar epithelial cells demonstrated cytoplasmic immunoreactivity for I κ B- β independent of the experimental regimen. The micrographs are representative of three independent experiments. Bars = 100 μ m.

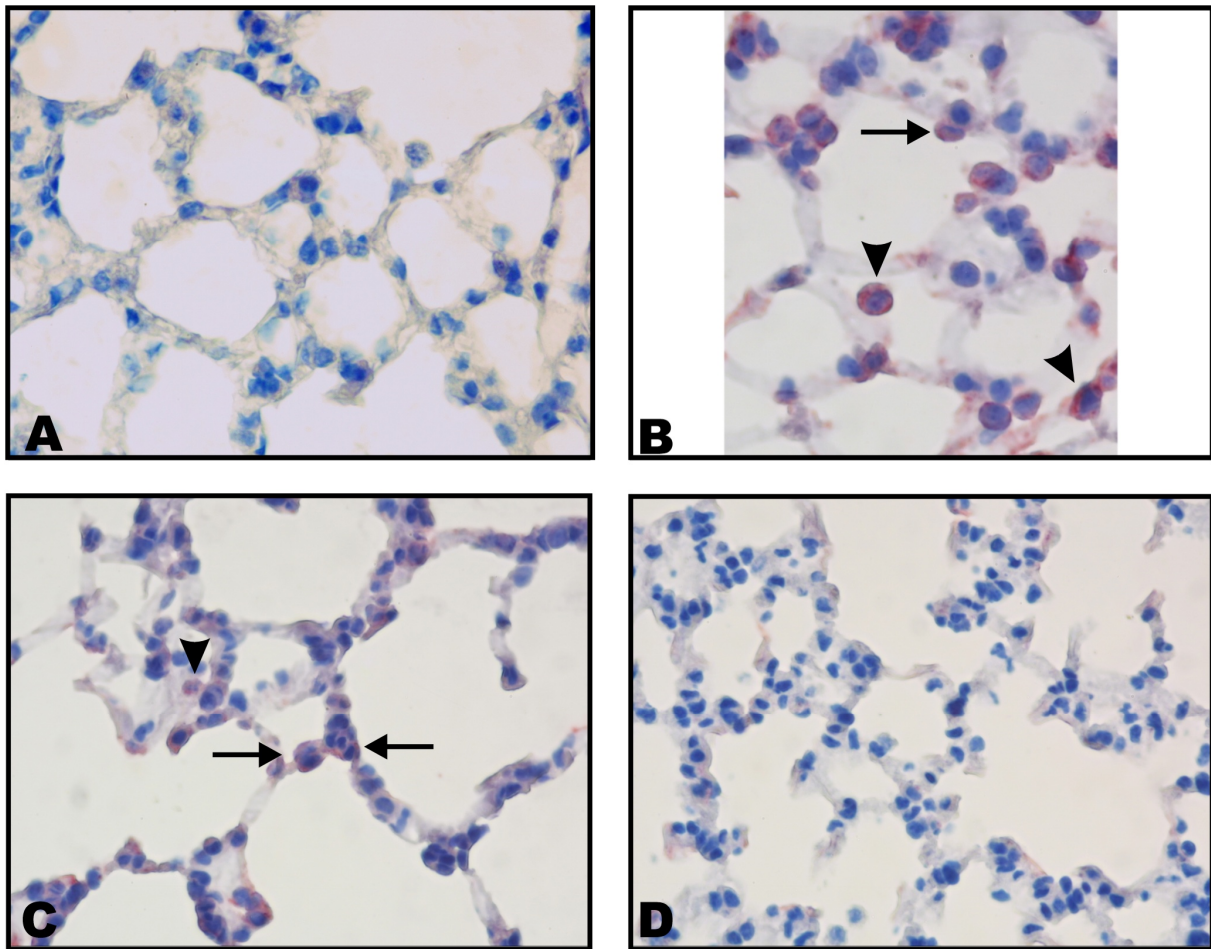


Figure 9. *In situ* hybridization for IL-6 mRNA in isolated perfused mouse lungs. Shown are micrographs from lungs that were ventilated with either -3/-10 cm H₂O EEP/EIP (control, panel **A**) or -3/-25 cm H₂O EEP/EIP (**B**, **C**) and overventilated lungs, which were treated with the PI3-kinase inhibitor Ly294002 (**D**). Cell types that after overventilation exhibited activation of IL-6 were identified as alveolar macrophages (arrows) and alveolar epithelial type II cells (arrowheads). The micrographs are representative of three independent experiments. Primary magnification 400x, except B (800x).

Cytokine release

In line with its effects on NF- κ B activation and IL-6 mRNA expression, treatment with Ly294002 prevented the ventilation-induced release of IL-6 and attenuated that of MIP-2 α . In contrast, Ly294002 had no effect on the release of these mediators if they were induced by LPS (Fig. 10). Please note that because of the lack of albumin in the perfusate buffer, the mediator levels were lower than previously reported⁸.

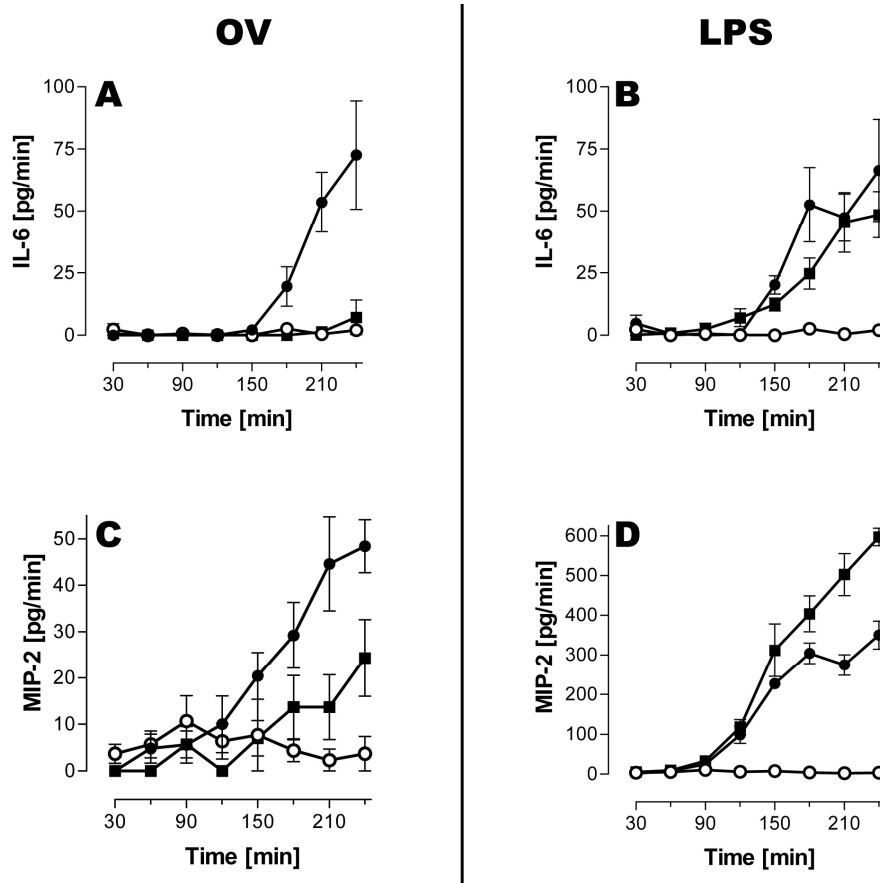


Figure 10. Differential inhibition of OV- and LPS-induced mediator production by inhibition of PI3K in isolated perfused mouse lungs. OV (overventilation, left) and LPS- (right)-induced alterations in IL-6 and MIP-2 α perfusate levels. Lungs were perfused for 60 min under control conditions (○, n=3) before they were exposed to either overventilation (n=5, ●, left panels A and C) or 50 μ g/ml LPS (n=3, ●, right panels B and D). 50 μ M Ly294002 were added 30 min before exposure to either OV (n=3, ■, left panels A and C) or LPS (n=4, ■, right panels B and D). IL-6 (upper panels A and B) and MIP-2 α (lower panels C and D) levels in the perfusate were assessed every 30 min.

Data are mean \pm SEM. In all panels, mediator release was significantly ($P < 0.05$) higher in overventilated or LPS-treated lungs compared to control perfused lungs. Pre-treatment with Ly294002 significantly ($P < 0.05$) reduced IL-6 and MIP-2 concentrations in overventilated, but not in LPS-treated lungs.

Discussion

Our previous finding that overventilation and endotoxin promote pro-inflammatory responses by different pathways that converge at the level of NF- κ B, suggested that it may be possible to block biotrauma without affecting innate immunity [8](#), [22](#). The present study shows that selective blockade of ventilation-induced mediator-release is indeed feasible, as shown by the differential effect of a PI3-kinase inhibitor on NF- κ B activation and mediator release triggered by OV and LPS, respectively, although for therapeutic application PI3-kinase inhibitors may have too many side effects (see below). Another key question concerns the cell types that are activated by overventilation. Here, we identify alveolar macrophages and alveolar type II cells as the major cell types in which NF- κ B is activated by overventilation.

Ventilation-induced mediator release can result from stress failure of cell membranes, stress failure of cell contacts, increased vascular shear stress or mechanotransduction processes triggered by stretch [35](#). The isolated perfused mouse lung model permits to study

relative selectively the latter mechanism in the whole intact organ, because increased vascular shear stress and stress failure are largely excluded by ventilation with negative pressure and end-expiratory pressure, respectively ³⁵. It should be noted that in isolated blood-free perfused mouse lungs the mode of overventilation applied does not cause acute lung injury, since none of the typical pathological changes such as edema or neutrophil influx occurs ^{10, 11, 47}. We therefore have speculated that the decrease in tidal volume is a result of surfactant exhaustion or airway derecruitment ^{10, 11}. The strength of the isolated perfused mouse lung is that it provides a well defined model to study the signaling events in intact overdistended alveoli, akin to what may happen in the ventilated and healthy parts of lungs with inhomogeneous lung injury ^{5, 35}. Many of the findings in perfused mouse lungs ^{8, 21} are reproduced in whole animals *in vivo*, as illustrated by the ventilation-induced release of MIP-2 α and KC in mice ¹⁸, the activation of NF- κ B and MAP kinases in rats ²¹ and the effect of Ly294002 on NF- κ B activation shown in this study (which was measured after 30 min, and thus too early for inflammation to occur).

Cells respond to external physical forces such as stretch, shear stress or UV light by well-defined intracellular responses that include stress activated protein kinases (SAPK), ion fluxes, NF- κ B and – as recently recognized – also PI3K. Activation of PI3K was demonstrated in endothelial cells by shear stress ^{48, 49} and in cardiac myocytes by stretch ⁵⁰. The present findings together with an accompanying study ³⁷ identify ventilation with high distending volumes/pressures as another physical force that activates PI3K. This was demonstrated by phosphorylation of Akt and by the effects of Ly294002 on activation of Akt, NF- κ B and mediator release. Ly294002 is a highly specific competitive inhibitor of the PI3K, binding to the ATP-binding site ⁵¹. Further evidence for the specificity of Ly294002 in our model is provided by its lack of effect on phosphorylation of other kinases such as Erk 1/2 and p38, and by the fact that it had no effect on LPS-induced NF- κ B activation or mediator release, a process that involves another set of kinases.

A large body of evidence now supports the concept that ventilation with high distending pressures triggers pro-inflammatory responses in the lung. However, the cell types which become activated to secrete those pro-inflammatory mediators have not been identified. Recently, we showed that overventilation elicits a PI3K-sensitive activation of Akt in pulmonary endothelial cells leading to production of NO ³⁷. This pattern of activation is clearly different from the staining pattern for overventilation-induced translocation of the NF- κ B p65 subunit, degradation of I- κ B β and enhanced expression of IL-6 mRNA, all of which occurred predominantly in alveolar type II epithelial cells and alveolar macrophages (Fig. 7

and 8, Fig. 9). This observation is in line with previous cell culture studies, showing stretch-dependent release of IL-8 from both cell types [6, 7](#). In addition, it was recently shown that ventilation with high pressures activated EMMPRIN, gelatinase A and gelatinase B in endothelial cells, alveolar type II epithelial cells and alveolar macrophages [52](#). All these findings suggest that in the whole organ both alveolar type II epithelial cells and alveolar macrophages contribute to the release of pro-inflammatory mediators elicited by overventilation. How these cells sense increased stretch (or more unlikely pressure) is still elusive, and thus we cannot exclude the possibility that alveolar macrophages and alveolar type II epithelial cells are activated only indirectly by products released from other cells or maybe even from nerve endings [53](#).

The mammalian Rel/NF- κ B transcription factor family is comprised of five homologous polypeptides, p50, p65, c-Rel, RelB, and p52. These subunits associate in a combinatorial fashion to form transcriptionally active homo- and heterodimers. The most prevalent and well-characterized species of NF- κ B dimer is the p50/p65 heterodimer. Therefore, it is not surprising that the p50/p65 dimer is also active in overventilated lungs (Fig. 7, Fig. 8 and 9). Nuclear translocation of the p50/p65 dimer is prevented by a family of transcription factor inhibitors, most important among them being I- κ B α and I- κ B β . Phosphorylation of these inhibitors triggers their degradation and frees NF- κ B to translocate to the nucleus. The present view is that I- κ B α regulates transient and I- κ B β persistent NF- κ B activation [54](#). Therefore, degradation of I- κ B β may be a critical event for the persistent NF- κ B activation observed in many disease states [54](#). Unfortunately, the molecular details of the regulation of I- κ B β are only poorly defined.

Expression of I- κ B β was diminished in alveolar cells of overventilated lungs and was restored by inhibition of PI3K, suggesting that PI3K leads to phosphorylation and degradation of I- κ B proteins. This is in line with other studies showing either activation of PI3K [50](#) or degradation of I- κ B β [55](#) by stretch in culture. At present, the mechanism linking PI3K to I- κ B β /NF- κ B during overventilation is unknown, although a number of recent studies have demonstrated a link between PI3K and I- κ B β /NF- κ B under a variety of different conditions (discussed previously in this article). The major pathways described involve direct effects on I- κ B or NF- κ B [33](#) or mediation by Akt or PKC isoenzymes. The different spatial activation of Akt in endothelial cells [37](#) and NF- κ B in alveolar macrophages and type II cells (Fig. 7, Fig. 8) suggests that Akt is not involved in the activation of NF- κ B by overventilation. However, since immunohistochemistry is a semiquantitative method, we cannot completely exclude that

limited activation of Akt may also occur in lung cells other than endothelial cells. An attractive alternative mechanism how PI3K may activate NF- κ B is activation of Ca²⁺-independent or atypical PKC isoforms by PtdIns^{3,4,5}P₃ [56-60](#). For instance, PKC- ζ , a target of PI3K [61, 62](#), can regulate NF- κ B through activation of I- κ B kinases [63, 64](#) or phosphorylation of the p65 subunit of NF- κ B [60, 62, 65](#).

Of particular interest is our observation that inhibition of PI3K did not prevent the NF- κ B activation and the cytokine release triggered by LPS. This is in line with previous studies in rat primary astrocytes [66](#), although controversial findings have been reported [67](#). Controversial results were also obtained with respect to LPS-induced activation of Akt in macrophages, showing phosphorylation in isolated human alveolar macrophages [68](#), but not the macrophage cell line RAW 264.7 [69](#). Thus at present, the role of PI3K and Akt during LPS stimulation in macrophages and type II cells is not well understood. However, given the redundancy of pathways activated by LPS [70](#), the failure of PI3K kinase inhibitors to affect LPS-induced NF- κ B activation may not be surprising.

Differing mechanisms of NF- κ B activation by LPS and OV were also suggested in our previous study, where in TLR-4-deficient mice NF- κ B was activated by overventilation, but not by LPS [8](#). In contrast to steroids, which block both LPS- and ventilation-induced NF- κ B activation and mediator release [8](#), PI3K-inhibition was selective for ventilation, suggesting that it may be possible to reduce some of the side effects of ventilation without causing severe immune suppression. However, the variety of cellular functions that are controlled by the PI3K pathway, including neutrophil activation [71](#), cell proliferation [72](#) or insulin receptor signaling [72](#), let it appear likely that blocking of this pathway might have marked side effects. Therefore, in future studies it will be important to establish the missing link between PI3K and NF- κ B, in order to define a more selective target. Alternatively, local (tracheal) administration of PI3K inhibitors might be considered as a way to reduce the side effects of PI3K inhibitors.

In summary, we have shown that overventilation triggers activation of NF- κ B in alveolar macrophages and alveolar epithelial type II cells. In line with its effect on NF- κ B and IL-6 mRNA expression, the PI3K inhibitor Ly294002 attenuated the ventilation-induced release of IL-6 and MIP-2 α suggesting alveolar macrophages and epithelial type II cells as sources of MIP-2 α and IL-6. The failure of Ly294002 to block LPS-induced responses suggests that it may be possible to selectively target the side effects of ventilation related to the release of pro-inflammatory mediators.

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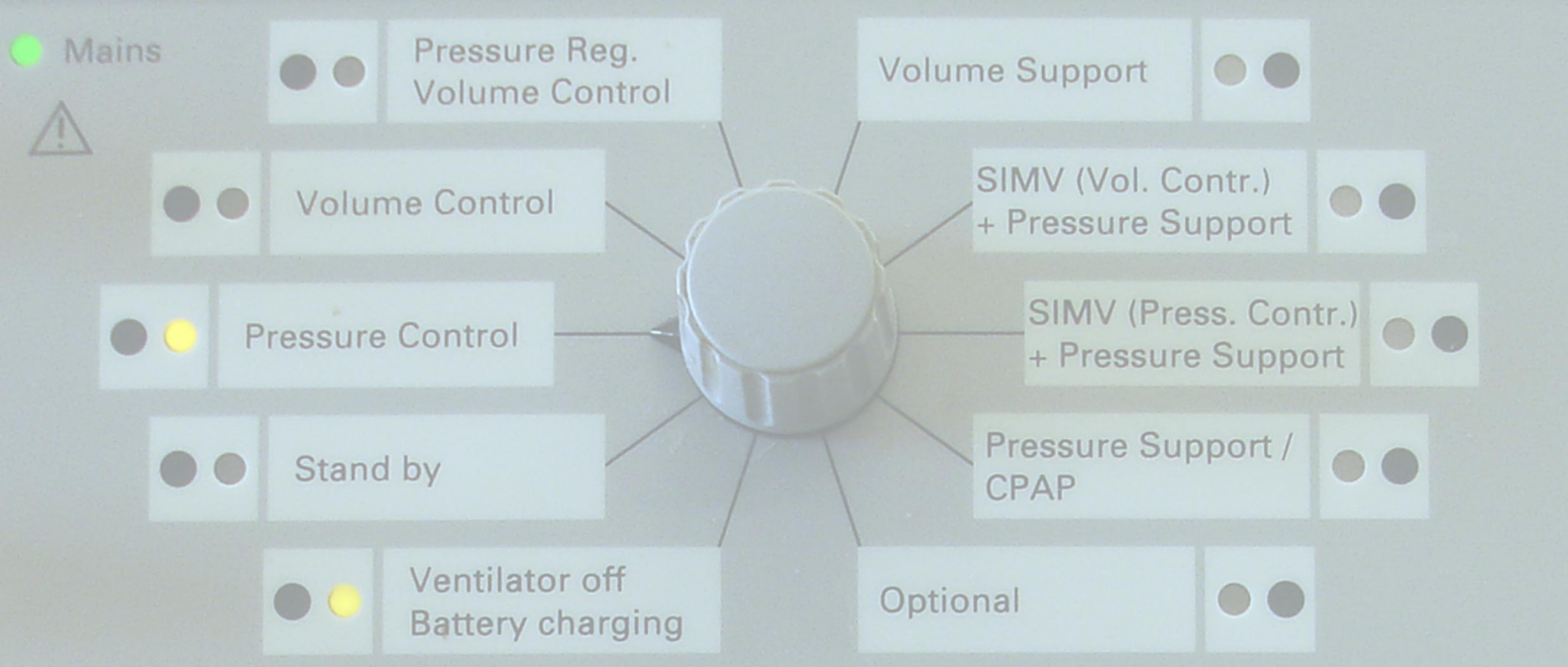
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Resp. pattern

Measured freq. b/min
50

Set freq. b/min
50

Insp. period s
0.40

Insp. flow l/s

Chapter 5

Reducing atelectasis attenuates bacterial growth and translocation in experimental pneumonia

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Abstract

Besides being one of the mechanisms responsible for ventilator-induced lung injury, atelectasis also seems to aggravate the course of experimental pneumonia. In this study, we examined the effect of reducing the degree of atelectasis by natural modified surfactant and/or open lung ventilation, on bacterial growth and translocation in a piglet model of Group B streptococcal pneumonia. After creating surfactant-deficiency by whole lung lavage, intratracheal instillation of bacteria induced severe pneumonia with bacterial translocation into the blood stream, resulting in a mortality rate of almost 80%. Treatment with 300 mg/kg of exogenous surfactant before instillation of streptococci attenuated both bacterial growth and translocation, and prevented clinical deterioration. This goal was also achieved by reversing atelectasis in lavaged animals via open lung ventilation. Combining both exogenous surfactant and open lung ventilation prevented bacterial translocation completely, comparable to Group B streptococci instillation into healthy animals. We conclude that exogenous surfactant and open lung ventilation attenuate bacterial growth and translocation in experimental pneumonia and that this attenuation is at least in part mediated by a reduction in atelectasis. These findings suggest that minimizing alveolar collapse by exogenous surfactant and open lung ventilation may reduce the risk of pneumonia and subsequent sepsis in ventilated patients.

Introduction

Pneumonia is a common finding in adult, pediatric and newborn patients admitted to the intensive care unit ¹⁻³. Its occurrence leads to an increased mortality rate, especially when complicated by severe sepsis or septic shock ⁴⁻⁶. Both mechanical ventilation and preceding colonization of the upper respiratory tract are considered important risk factors in the development of pneumonia ^{1-3, 7}. The precise mechanisms responsible for the progression from colonization to pneumonia and more importantly to sepsis remain unclear. Animal studies have shown that alveolar macrophages (AMs), which are considered the first line of host defense against organisms entering the lower respiratory tract ⁸, play an essential role in bacterial clearance and survival in experimental pneumonia ^{9, 10}. In contrast to AMs, the role of pulmonary surfactant in the development of pneumonia is much less clear. Besides several non-specific defense mechanisms, the main effect of pulmonary surfactant on host defense is attributed to the surfactant proteins (SP)-A and SP-D ¹¹.

The contribution of the biophysical properties of pulmonary surfactant, i.e. lowering the alveolar surface tension and thus preventing alveolar collapse and edema, to the host defense of the lung has not been extensively explored. The degree of atelectasis might prove important as atelectrauma is considered one of the important mechanisms responsible for the development of ventilator-induced lung injury, and previous animal studies showed that reducing atelectasis by positive end-expiratory pressure (PEEP) mitigates both bacterial growth in the lung and translocation from the lung into the blood stream ¹²⁻¹⁴.

We therefore hypothesized that exogenous surfactant would enhance bacterial clearance from the lung and attenuate systemic bacterial dissemination in experimental pneumonia, and that this effect is at least in part mediated by a reduction in the degree of atelectasis.

To test this hypothesis we induced experimental pneumonia in newborn piglets by intratracheal injection of group B streptococci (GBS), which are the leading cause of serious infections in human newborns and are of growing importance in invasive infections in adults ^{15, 16}. Using whole lung lavage and exogenous surfactant, we created different conditions of the pulmonary surfactant system. We used natural modified surfactant containing only phospholipids and hydrophobic surfactant proteins (SP-B, SP-C), because this type of surfactant is frequently used in daily clinical practice and proved to be superior to synthetic preparations not containing SP-B and SP-C ¹⁷. Furthermore, natural modified surfactant does

not contain SP-A and SP-D, which enabled us to make a more valid assessment of the effect of reducing atelectasis on bacterial growth and translocation in experimental pneumonia.

To elucidate the role of atelectasis further, additional groups of both surfactant sufficient and deficient animals were subjected to open lung ventilation aiming to recruit collapsed alveoli and prevent subsequent atelectasis by applying sufficient PEEP (open lung concept, OLC) [18](#).

Besides assessing the effects of these different interventions on bacterial growth in the lung and bacterial translocation to the bloodstream, we also measured the effects on survival and severity of lung injury.

Some of the results of these studies have been previously reported in the form of an abstract [19, 20](#).

Methods

Bacteria

An encapsulated GBS Ia 90 LD serological subtype was used in the present study, as preliminary experiments showed that this strain was able to induce severe pneumonia in newborn piglets. Aliquots of GBS stored at -70°C were inoculated in 12 ml trypticase soy broth (TSB 3%, Becton Dickinson, Cockeysville, MD) and kept at 37°C for 16 hours. From this solution, 8 ml was inoculated into 50 ml fresh trypticase soy broth and incubated at 37°C for 3 hours in order to shift bacterial growth to the mid logarithmic phase. The bacteria were then centrifugated, washed and resuspended in sterile saline at a concentration of approximately 10 CFU per ml as determined by spectrophotometric measurement of the OD at 595 nm. The exact number of viable CFU/ml in the GBS suspension was determined by serial dilution as previously described [21](#).

Animal preparation

Anesthesia was induced in 81 mixed-breed newborn piglets, aged 74 ± 16 (SD) hours and weighing 2.0 ± 0.4 (SD) kg, with ketamine hydrochloride (35 mg/kg, intramuscularly) and midazolam (0.5 mg/kg, intramuscularly). The animals were tracheotomized and thereafter ventilated in the pressure controlled time-cycled mode (Servo 300 Siemens-Elema, Solna, Sweden) at a fractional inspired oxygen concentration of 1.0, PEEP of 2 cmH₂O, a rate of 25-30 breaths/minute and an inspiratory/expiratory ratio of 1:2.

Tidal volume was measured at the Y-piece using a stand-alone respiratory monitor (CO₂SMO Plus, Novamatrix Systems, Wallingford, CT). Peak inspiratory pressures (PIP) were adjusted to keep the expiratory tidal volumes between 7-10 ml/kg. A neuromuscular block was induced with pancuronium bromide (0.5 mg/kg, intravenously), followed by a continuous infusion of fentanyl (20 µg/kg/h), midazolam (0.3 mg/kg/h) and pancuronium bromide (0.3 mg/kg/h).

Using an aseptic technique, catheters were inserted into the external jugular vein for measurement of central venous pressure and infusion of fluids and medication, and the carotid artery for monitoring of blood pressure and blood sampling.

A continuous infusion of 5% dextrose was started (100 ml/kg/d) and body temperature was kept between 38°C and 39°C during the experiments.

Lavage procedure

In a subgroup of animals respiratory failure was induced by removing the endogenous surfactant through repeated saline lavage (50 ml/kg; 37°C) as previously described [22](#). Lavages were repeated at 3-minute intervals until PaO₂ was below 80 mmHg and PaCO₂ above 40 mmHg at the following ventilator settings: PIP/PEEP 20/5 cmH₂O, rate 30 breaths/minute, inspiratory/expiratory ratio 1:2 and a fractional inspired oxygen concentration of 1.0. The time needed for the lavage procedure was approximately 45 minutes.

Surfactant treatment

Animals treated with exogenous surfactant received a dose of 300 mg/kg natural modified surfactant suspended in saline at a concentration of 50 mg/ml. The surfactant was administered as a bolus directly into the endotracheal tube. The surfactant (HL 10, Leo Pharmaceutical Products, Ballerup, Denmark; Halas Pharma GmbH, Oldenburg, Germany) used was isolated from minced pig lungs as previously described [23](#).

This surfactant contains 98% lipids (mainly phospholipids) and 1-2% hydrophobic proteins SP-B and SP-C. Animals not treated with exogenous surfactant received an equal volume (6 ml/kg) of air.

GBS instillation

Thirty minutes after the administration of either surfactant or air bolus, the animals received 10 ml/kg of the GBS suspension. This suspension was slowly injected through a catheter placed at the end of the endotracheal tube. To ensure equal distribution, the first 5 ml/kg were

injected with the animal placed on its right side. After 5 minutes of ventilation the animal was turned to the left side and a second dose of 5 ml/kg was injected in a similar fashion. Following this procedure the animals were returned to the supine position for the remainder of the experiments.

Ventilation strategies

All animals received positive pressure ventilation, but depending on the treatment group they were subjected to one of two ventilation strategies. Fractional inspired oxygen concentration was kept at 1.0 during all experiments.

Conventional positive pressure ventilation (PPV_{CON}). During PPV_{CON} animals were ventilated in the pressure controlled mode. The PIP was set at a level that resulted in an expiratory tidal volume of approximately 7 ml/kg. The level of PEEP was maximized at 5 cmH₂O and the ventilatory rate could be adjusted between 30-60 breaths/min in order to prevent hypercapnia (PaCO₂ > 55 mmHg).

Open lung concept positive pressure ventilation (PPV_{OLC}). As previously described, the main objective of this ventilation strategy is to recruit atelectatic lung regions and prevent repeated alveolar collapse during expiration²². We used changes in intrapulmonary shunt and subsequent changes in oxygenation as an indirect tool to assess the degree of alveolar collapse. For this reason, a sensor for continuous blood gas monitoring (Paratrend, Diametrics Medical Ltd., Buckinghamshire, UK) was inserted through a femoral artery catheter. Based on PaO₂ levels in the healthy piglets ventilated with a fractional inspired oxygen concentration of 1.0, we defined optimal alveolar recruitment when PaO₂ ≥ 450 mmHg. Immediately after randomization to the PPV_{OLC} group, PEEP was increased to 10 cmH₂O and the pressure amplitude (PIP minus PEEP) was set at 10-12 cmH₂O. Hypercapnia (PaCO₂>55 mmHg) was prevented by increasing the ventilatory rate to 100 breaths/minute. Preliminary experiments showed no intrinsic PEEP at these settings. During the experiments expiratory flow was observed to be zero prior to each inspiration. Collapsed alveoli were then recruited by increasing both PEEP and PIP in steps of 2 cmH₂O every 2 minutes, until the PaO₂ > 450 mmHg. The level of PIP needed to recruit the lung was called the opening pressure (PIP_O). After this recruitment procedure PEEP and PIP were simultaneously decreased in steps of 2 cmH₂O every 2 minutes until PaO₂ dropped below 450 mmHg, indicating increased intrapulmonary shunt due to alveolar collapse. The level of PEEP at this stage of alveolar collapse was called the closing pressure (PEEP_C). Collapsed alveoli were once again recruited with the known PIP_O and PEEP was set at a level of 2 cmH₂O above

PEEP_C. As the lung was now ventilated on the more compliant deflation limb of the pressure-volume (P/V) curve, the pressure amplitude was reduced as much as possible, keeping PaCO₂ within the target range.

Hemodynamic support

Hemodynamic deterioration was defined as: 1) a decrease mean arterial blood pressure of more than 10% and; 2) a heart rate > 200 beats/minute or an increase of more than 10% if baseline values were already above this threshold value. We used the mean arterial blood pressure and heart rate during the first hour of ventilation after GBS instillation as baseline values. If both criteria were met, intravascular volume expansion was administered, using sterile saline with a maximum cumulative dose of 50 ml/kg. In addition, dopamine was started after administration of the first 20 ml/kg saline, in a dose of 20 µg/kg/min.

Experimental groups

After the instrumentation period the animals were randomly allocated to one of the following groups. All groups consisted of 13 animals, unless stated differently. The ventilation period after GBS instillation was 5 hours, except in the growth control group.

Growth controls. A total of 8 ventilated animals (4 lavaged, 4 healthy) received 10 ml/kg of the GBS suspension according to the procedure described above. After 5 minutes of ventilation the animals were killed and the lungs were aseptically removed. Total, left and right lung weights were recorded, after which the number of GBS was determined in both the left and right lung.

The total number of CFU/lung was calculated by using the data of the left lung, and using the data of both the left and the right lung. These data were used to check viability and distribution of the bacteria immediately after inoculation.

Healthy. Animals allocated to this group were ventilated for 45 minutes after the instrumentation period in order to synchronize the ventilation time with animals subjected to lung lavage. After this ventilation period the animals received an intratracheal bolus of air and 30 minutes later the GBS solution was injected via the same route. Following GBS instillation animals were ventilated according to the PPV_{CON} strategy.

Lavaged. After the instrumentation period animals in this group were subjected to lung lavage followed by an intratracheal bolus of air. Thirty minutes later the GBS suspension was instilled and animals were ventilated according to the PPV_{CON} strategy.

Surfactant. Animals in this group received an intratracheal bolus of exogenous surfactant after the lavage procedure, followed by GBS instillation 30 minutes later. Ventilation was according to the PPV_{CON} strategy.

OLC. Animals in this group were subjected to the same procedures as the *laved* group but were ventilated according to the PPV_{OLC} strategy.

Surfactant-OLC. As in the *surfactant* group, animals in this group received exogenous surfactant after the lavage procedure but were ventilated according to the PPV_{OLC} strategy after GBS instillation.

Saline. The 8 animals in this control group, received a bolus of air after lung lavage but, instead of the GBS solution, 10 ml/kg of sterile saline was instilled in a similar fashion. Ventilation was according to the PPV_{CON} strategy.

Data acquisition and outcome parameters

Ventilation and hemodynamics. Ventilatory parameters (airway pressures, tidal volumes, ventilatory rates) and hemodynamic parameters (mean arterial blood pressure, central venous pressure, heart rate) were recorded after the instrumentation period, after lung lavage, after surfactant/air administration and every 15 minutes after GBS instillation.

Blood gas analysis. Samples for blood gas analysis (ABL 555, Radiometer, Copenhagen, Denmark) were drawn at the end of the instrumentation period, after lung lavage, after surfactant/air bolus and hourly after GBS instillation.

CFU in blood. Blood cultures were drawn under aseptic conditions at the end of the instrumentation period, just prior to the instillation of the GBS solution and hourly thereafter. The number of CFU/ml blood was calculated by spreading 1 ml of whole blood on a blood agar plate (Becton Dickinson, Alphen a/d Rijn, The Netherlands), which was incubated for 24 hours at 37°C.

Survival. Survival time starting after GBS instillation was recorded for all animals. Those animals still alive after the 5 hours ventilation period were killed using an overdose of pentobarbital.

Lung function. In five animals in the *saline* groups and ten animals in the other groups, the thorax and diaphragm was opened immediately after death and P/V curves were constructed using the syringe technique, as previously described [22](#). Maximal lung compliance was calculated from the steepest slope on the deflation limb. Lung volumes at

transpulmonary pressures of 35 (total lung capacity) and 5 cmH₂O were recorded from the deflation limb.

CFU in lung homogenate. In animals subjected to lung function measurements the lungs were aseptically removed and weighed after removing the heart and great vessels. After clamping the left main bronchus, the left lung was dissected, weighed and after adding 25 ml sterile saline, homogenized at 4°C for 2 minutes at 40,000 rpm in a tissue homogenizer (Virtis “23”, The Virtis Company Inc., NY) ²⁴. The volume of the lung homogenate was recorded and a 1 ml aliquot was serially diluted and spread on blood agar plates for calculation of the number of viable CFU/ml homogenate. Using the data on homogenate volume, left lung weight and total lung weight, the number of CFU/lung was calculated.

Broncho-alveolar lavage (BAL). After dissecting the left lung, BAL of the right lung was performed five times (40 ml/kg) with saline solution supplemented with 1.2 mM CaCl₂. The recovered fluids from these five lavages were pooled and analyzed as one sample. The percentage of lung lavage fluid recovered was calculated. Samples were centrifugated for 10 minutes at 1500 g to remove cell material. Protein concentration was measured using the Bradford method (Biorad protein assay, Munich, Germany) ²⁵. SP-A was measured by ELISA using purified rabbit and chicken antibodies, which were specific for porcine SP-A. After a peroxidase reaction, absorbance was read at 450 nm. SP-A levels in BAL fluid were expressed as µg/ml BAL fluid.

Histology. Three animals in each group were used for histological analysis as previously described ²². Briefly, after perfusion the lung was fixated with a solution consisting of 3.6% formaldehyde and 0.25% glutaraldehyde. Prior to fixation, the airway pressure was increased to 30 cmH₂O for 15 sec, and thereafter maintained at 20 cmH₂O for the remainder of the fixation process. Blocks of tissue were taken from the center of the upper and middle and lower lobes of the right lung.

The specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin. The presence of bacteria, the influx of inflammatory cells, edema and hyaline membranes were semi-quantatively scored as none, minimal, light, moderate or severe (score 0, 1, 2, 3 or 4, respectively) taking into account both the severity and the extent.

The total score for each variable was defined as the sum of all three lobes (maximum score 12). The lung injury score for each treatment group was obtained by averaging the scores from the included animals. Scoring was done by one of the authors (A.N.) under blinded conditions.

Statistical analysis. All data are expressed as mean \pm SD. Data on bacterial growth were subjected to logarithmic transformation (\log_{10}). Statistical analysis was performed using SPSS version 11 (SPSS Chicago, IL, USA). Intergroup differences were analyzed with the analysis of variance followed by a Bonferroni post-hoc test. Pearson's correlation and χ^2 test were used when appropriate. The Kaplan Meier analysis followed by a log rank test was used to compare survival and bacterial translocation. A p -value of 0.05 or less was considered statistically significant.

Results

Animals

A total of 81 animals were included with a mean age of 74 ± 16 (SD) hours and weighing 2.0 ± 0.4 kg. There were no intergroup differences in age, weight, or number of lavages needed to induce lung injury. No air leaks were observed during the study period.

Growth controls

Figure 1 shows that the viability of the GBS bacteria in the lung 5 minutes after intratracheal injection was similar to that of the GBS solution. Furthermore, there was an excellent correlation ($r = 0.97$, $p < 0.001$) between the number of CFU/lung calculated on the basis of the left lung and both the left and the right lung, indicating an even distribution of the GBS solution between the right and left lung after intratracheal injection.

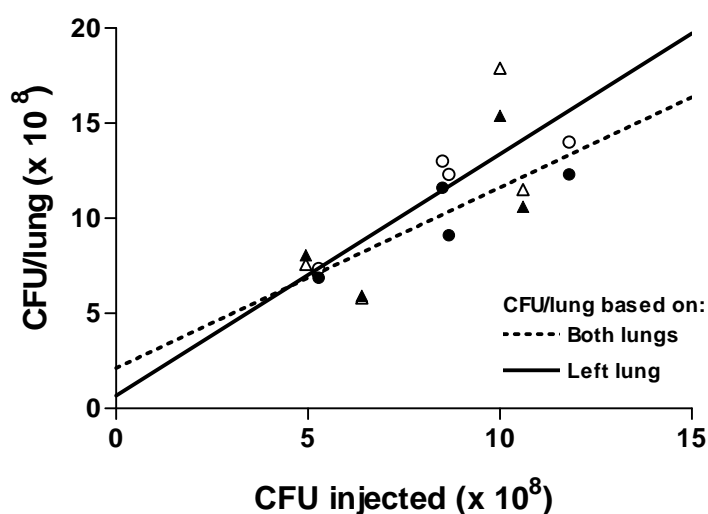


Figure 1. Correlation between the number of CFU injected intratracheally and the number of CFU isolated from the lung. Calculation of the total number of CFU in the lung was based on the left lung (open symbols; $r = 0.79$, $p < 0.05$) and the left and right lung (closed symbols; $r = 0.76$, $p < 0.05$). Animals were either healthy (circles) or lavaged (triangles).

Survival

Eleven of the 13 animals in the *lavaged* group died during the ventilation period, with a mean survival time of 211 ± 49 minutes (Figure 2). This was significantly different from the other groups, where all animals survived the 5-hour ventilation period.

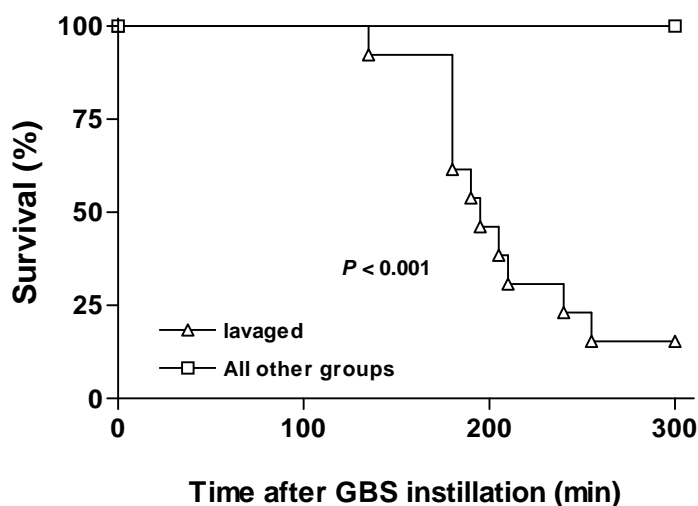


Figure 2. Survival plots for the different groups after GBS instillation into the airways. *Lavaged*, *lavaged+GBS+PPV_{CON}*.

CFU in lung homogenate

The number of CFU/lung instilled intratracheally was similar in all intervention groups (Figure 3). The number of CFU/lung decreased after 5 hours of ventilation in the *healthy* group ($p < 0.001$), and remained stable in the *OLC* and *surfactant-OLC* groups. In the *lavaged* and the *surfactant* group the number of CFU/lung increased over time ($p < 0.001$ and $p < 0.01$, respectively), but this growth was significantly less in the *surfactant* group.

The total lung weight corrected for body weight at the end of the experiments was significantly higher in the *lavaged* group compared to all other groups (Table 1). The lung weight in the *surfactant* group was significantly higher compared to the *healthy*, *OLC* and *surfactant-OLC* groups. There were no differences between these latter three groups.

CFU in blood

None of the animals had positive blood cultures prior to GBS instillation. As shown in Figure 4, blood cultures remained negative throughout the ventilation period for all animals in the *healthy* and the *surfactant-OLC* groups.

In the *lavaged* group all except one animal had GBS positive blood cultures, with a mean time to bacteremia of 97 ± 18 minutes. The use of surfactant or OLC ventilation resulted in a comparable reduction in the number of animals with GBS positive blood cultures (7/13 and

6/13, respectively) and the time to bacteremia (291 ± 6 and 245 ± 23 minutes, respectively). The maximum number of CFU/ml blood was also significantly higher in the *lavaged* group (265 ± 165) compared with the *surfactant* group (6 ± 5 , $p < 0.001$) and the *OLC* group (33 ± 53 , $p < 0.005$).

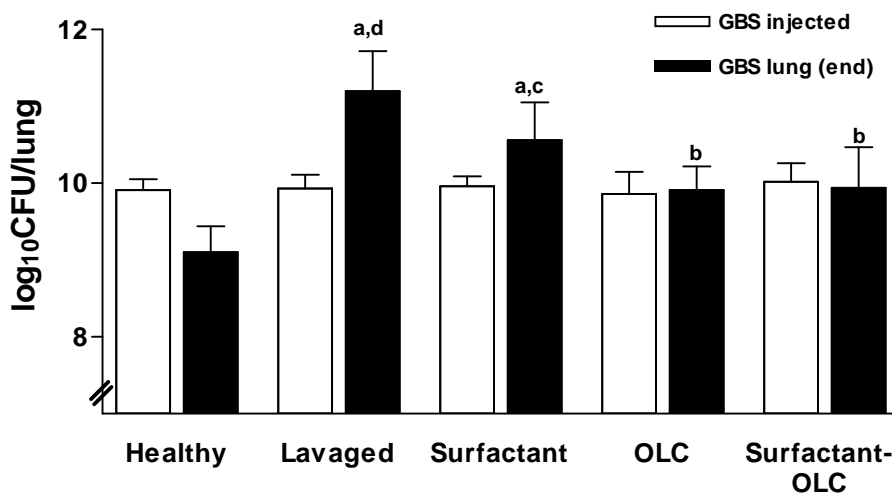


Figure 3. The initial number of CFU (mean \pm SD) expressed as \log_{10} CFU/lung, injected in the lung and the subsequent proliferation during the ventilation period. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. ^a $p < 0.001$, ^b $p < 0.005$ vs healthy. ^c $p < 0.05$ vs lavaged, OLC and surfactant-OLC. ^d $p < 0.001$ vs OLC and surfactant-OLC.

Gas exchange

PaO₂ or PaCO₂ levels after the instrumentation period and after lung lavage were comparable in the different groups (Figure 5). In the *lavaged* group, oxygenation deteriorated over time as animals developed severe pneumonia (Figure 5A). Adding surfactant significantly improved oxygenation. In both groups ventilated with PPV_{OLC}, PaO₂ levels returned to prelavage values and this was maintained throughout the ventilation period, indicating successful application of the open lung approach. Except for higher PaCO₂ levels in the *lavaged* group, the PaCO₂ levels were comparable between the different groups after GBS or saline instillation (Figure 5B).

Ventilatory and circulatory parameters

There were no differences in ventilatory and circulatory parameters between the different groups prior to and immediately after lung lavage. As expected, the mean airway pressure and PEEP was higher in the groups ventilated with PPV_{OLC} compared to the PPV_{CON} groups (Table 2). The mean expiratory tidal volume during PPV_{CON} was within the target range, and slightly below this range during PPV_{OLC} (Table 2).

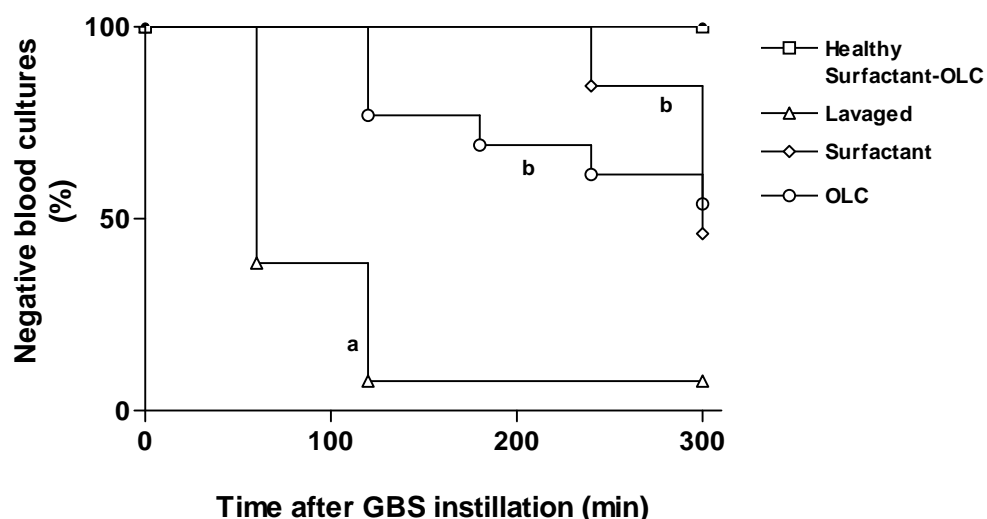


Figure 4. Kaplan-Meier curves displaying the percentage of animals in each group with negative blood cultures during the ventilation period. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. ^a $p < 0.001$ vs all other groups. ^b $p < 0.01$ vs healthy and surfactant-OLC.

In contrast to the other groups, the mean arterial blood pressure from animals in the *lavaged* group deteriorated over time, which was accompanied by an increase in heart rate (Table 3). In accordance with these findings, 12 animals in the *lavaged* group compared to less than 2 animals in the other groups required intravascular volume support ($p < 0.001$) and dopamine infusion ($p < 0.001$).

Lung function

Pressure-volume curves constructed postmortem showed a severe deterioration of lung function in the *lavaged* group (Table 1). Surfactant therapy attenuated this deterioration, but not completely. There were no differences between the other groups.

Proteins in BAL

Alveolar protein influx was most severe in the *lavaged* group and although surfactant therapy reduced protein influx to some extent, this difference was not statistically significant (Table 1). The recovery of BAL fluids was not different among the groups (data not shown).

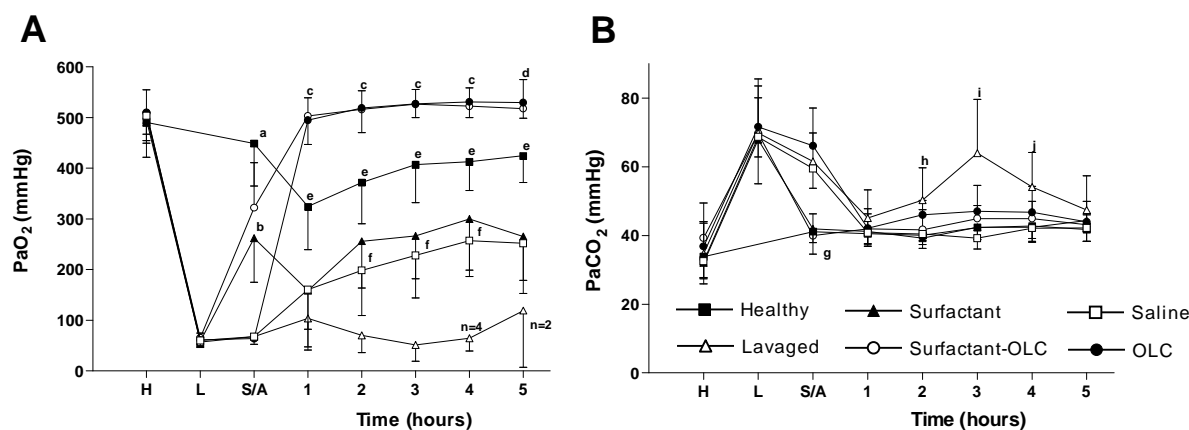


Figure 5. Changes (mean \pm SD) in PaO₂ (A) and PaCO₂ (B) levels in the six treatment groups. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. *Saline*, lavaged + saline + PPV_{CON}. Values represent changes before (H), after lavage (L), after surfactant or air bolus (S/A), and during the 5-h ventilation period after GBS instillation. The number of animals still alive in the *lavaged* group at time points 4 and 5 hours are indicated. ^a $p < 0.001$ vs all other groups. ^b $p < 0.001$ surfactant and surfactant-OLC vs lavaged, OLC and saline. ^c $p < 0.001$ OLC and surfactant-OLC vs all other groups. ^d $p < 0.001$ OLC and surfactant-OLC vs lavaged, surfactant and saline, and $p < 0.05$ vs healthy. ^e $p < 0.001$ vs lavaged, surfactant and saline. ^f $p < 0.001$ surfactant and saline vs lavaged. ^g $p < 0.001$ healthy, surfactant and surfactant-OLC vs lavaged, OLC and saline. ^h $p < 0.005$ lavaged vs healthy, surfactant, surfactant-OLC and saline. ⁱ $p < 0.001$ vs all other groups. ^j $p < 0.01$ lavaged vs healthy, surfactant and saline.

Table 1. Data (mean \pm SD) on lung weight, lung mechanics and alveolar protein influx

	Healthy	Lavaged	Surfactant	OLC	Surfactant-OLC	Saline
WW/BW* g/kg	30 \pm 3	52 \pm 8 ^a	45 \pm 6 ^b	37 \pm 3	34 \pm 3	31 \pm 2
C _{Lmax} [†] ml/cmH ₂ O/kg	5.4 \pm 1.4	1.2 \pm 0.7 ^a	3.1 \pm 1.2 ^b	5.0 \pm 1.6	5.9 \pm 1.3	6.0 \pm 1.3
TLC ₃₅ [‡] ml/kg	57 \pm 20	22 \pm 6 ^a	44 \pm 14 ^c	59 \pm 15	60 \pm 11	67 \pm 6
V _s [§] ml/kg	38 \pm 15	10 \pm 4 ^d	23 \pm 10 ^e	39 \pm 11	39 \pm 7	45 \pm 4
Protein mg/ml	0.49 \pm 0.21	1.38 \pm 0.57 ^f	1.03 \pm 0.50 ^g	0.79 \pm 0.17	0.90 \pm 0.10	0.56 \pm 0.33
SP-A pg/ml	80 \pm 43	77 \pm 45	54 \pm 29	70 \pm 37	85 \pm 42	85 \pm 21

* wet weight lung weight to body weight ratio. [†] maximal lung compliance. [‡] and [§], lung volumes at a transpulmonary pressure of respectively, 35 (total lung capacity) and 5 cmH₂O. ^{||}, surfactant protein A. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. *Saline*, lavaged + saline + PPV_{CON}. ^a $p < 0.005$ vs healthy, OLC, surfactant-OLC and saline, and $p < 0.05$ vs surfactant. ^b $p < 0.005$ vs healthy, surfactant-OLC and saline, and $p < 0.05$ vs surfactant and OLC. ^c $p < 0.05$ vs lavaged and saline. ^d $p < 0.005$ vs healthy, OLC, surfactant-OLC and saline. ^e $p < 0.005$ vs saline and $p < 0.05$ vs healthy, OLC and surfactant-OLC. ^f $p < 0.005$ vs healthy and saline, and $p < 0.05$ vs OLC. ^g $p < 0.05$ vs healthy

SP-A in BAL

SP-A levels measured in BAL obtained at the end of the ventilation period in the *healthy*, *lavaged*, *surfactant*, *OLC*, *surfactant-OLC* and *saline* groups were detectable in respectively, 50%, 30%, 50%, 40%, 40% and 40% of the animals. As shown by Table 1, mean SP-A content was not significantly different between the groups.

Table 2. Ventilatory parameters (mean \pm SD) over time in the groups

	Healthy	Lavaged	Surfactant	OLC	Surfactant-OLC	Saline
MawP* (cmH ₂ O)						
1 h	8.2 \pm 0.4 ^a	11.1 \pm 1.3 ^b	9.3 \pm 1.2 ^c	14.5 \pm 1.4	13.2 \pm 0.8	10.3 \pm 1.4
3 h	8.2 \pm 0.7 ^c	12.2 \pm 1.7 ^d	9.4 \pm 1.1 ^c	14.6 \pm 1.3	13.2 \pm 0.8	9.8 \pm 1.8
5 h	8.4 \pm 0.5 ^{ef}	10.5 \pm 0.7 ^g	9.8 \pm 1.2 ^{hf}	13.2 \pm 1.6	10.9 \pm 1.0	9.3 \pm 0.9
PEEP[†] (cmH ₂ O)						
1 h	4.0 \pm 0.0 ^e	4.4 \pm 0.7 ^e	4.1 \pm 0.2 ^e	10.6 \pm 1.0	10.0 \pm 0.6	4.0 \pm 0.0 ^e
3 h	4.0 \pm 0.0 ^e	4.8 \pm 0.9 ^e	4.0 \pm 0.0 ^e	10.5 \pm 1.1	9.6 \pm 0.9 ^g	4.0 \pm 0.0 ^e
5 h	4.0 \pm 0.0 ^e	4.0 \pm 0.0 ^e	4.0 \pm 0.0 ^e	8.8 \pm 1.4	7.5 \pm 1.0 ^g	4.0 \pm 0.0 ^e
PA[‡] (cmH ₂ O)						
1h	10.7 \pm 1.0 ^b	19.2 \pm 2.7 ⁱ	14.2 \pm 2.9 ^j	7.3 \pm 1.0	7.1 \pm 0.6	17.0 \pm 3.2 ^k
3h	11.1 \pm 1.0 ^{lg}	21.4 \pm 3.8 ^b	14.3 \pm 2.9 ^j	8.1 \pm 0.8	7.4 \pm 0.7	15.5 \pm 2.8 ^j
5h	12.8 \pm 1.5 ^m	17.0 \pm 0.5 ^e	16.5 \pm 3.6 ^k	8.9 \pm 0.9	7.6 \pm 1.0	14.2 \pm 2.2 ^e
VT_{Ex}[§] (ml/kg)						
1 h	7.5 \pm 0.9 ^e	7.7 \pm 0.9 ^e	7.4 \pm 0.6 ^e	6.0 \pm 0.6	6.2 \pm 0.5	7.5 \pm 0.3 ^e
3 h	6.9 \pm 0.7 ^h	6.7 \pm 0.8 ^g	6.7 \pm 0.5 ^g	6.0 \pm 0.5	6.3 \pm 0.5	7.0 \pm 0.3 ^h
5 h	7.0 \pm 1.0 ^g	7.7 \pm 0.1	6.5 \pm 0.6	6.3 \pm 0.4	6.4 \pm 0.4	6.8 \pm 0.4

* mean airway pressure. † positive end-expiratory pressure. ‡ pressure amplitude. § expiratory tidal volume. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. *Saline*, lavaged + saline + PPV_{CON}. ^a $p < 0.005$ vs lavaged, OLC, surfactant-OLC and saline. ^b $p < 0.005$ vs all other groups. ^c $p < 0.005$ vs lavaged, OLC and surfactant-OLC. ^d $p < 0.005$ vs healthy, surfactant, OLC and saline. ^e $p < 0.005$ vs OLC and surfactant-OLC. ^f $p < 0.05$ vs surfactant. ^g $p < 0.05$ and ^h $p < 0.005$ vs OLC. ⁱ $p < 0.005$ vs healthy, surfactant, OLC and surfactant-OLC. ^j $p < 0.005$ vs healthy, lavaged, OLC and surfactant-OLC. ^k $p < 0.005$ vs healthy, OLC and surfactant-OLC. ^l $p < 0.005$ vs lavaged, surfactant, surfactant-OLC and saline. ^m $p < 0.005$ vs surfactant, OLC and surfactant-OLC.

Histology

The histology findings were consistent with the other outcome parameters showing relatively mild abnormalities in the healthy animals after 5 hours of ventilation compared to signs of severe pneumonia in the *lavaged* group (Table 4). Treatment with either exogenous surfactant or OLC ventilation significantly mitigated histological severity of pneumonia.

Table 3. Circulatory parameters (mean \pm SD) over time in the groups

	Healthy	Lavaged	Surfactant	OLC	Surfactant- OLC	Saline
MAP* (mmHg)						
1 h	67 \pm 9	69 \pm 7	65 \pm 6 ^a	76 \pm 9	75 \pm 15	69 \pm 9
3 h	63 \pm 5 ^b	47 \pm 21 ^c	64 \pm 5	71 \pm 9	65 \pm 12	65 \pm 4
5 h	60 \pm 3	54 \pm 27	65 \pm 8	68 \pm 9	63 \pm 10	72 \pm 9
CVP† (mmHg)						
1 h	6.4 \pm 0.9 ^d	6.8 \pm 1.5 ^d	7.5 \pm 1.3 ^e	10.1 \pm 1.0	9.5 \pm 2.5	8.7 \pm 2.0 ^f
3 h	5.5 \pm 1.4 ^d	6.9 \pm 1.7	7.1 \pm 1.1	8.5 \pm 0.8	8.2 \pm 2.0	7.4 \pm 1.1
5 h	5.3 \pm 0.9 ^d	4.8 \pm 1.0	6.9 \pm 1.0	7.9 \pm 0.8	7.5 \pm 2.1	6.9 \pm 1.8
HR‡ (beats/min)						
1 h	165 \pm 29 ^{eg}	198 \pm 36	174 \pm 30 ^a	217 \pm 18	208 \pm 55	179 \pm 37
3 h	164 \pm 27 ^h	228 \pm 37 ^{ij}	188 \pm 22 ^{abg}	232 \pm 35	236 \pm 25	181 \pm 34 ^{abg}
5 h	178 \pm 35 ^{bdk}	251 \pm 43 ^f	226 \pm 35 ^j	251 \pm 22	244 \pm 32	196 \pm 31 ^{eg}

* mean arterial pressure. † central venous pressure. ‡ heart rate. *Healthy*, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. *Saline*, lavaged + saline + PPV_{CON}. ^a $p < 0.05$ vs OLC. ^b $p < 0.05$ vs lavaged. ^c $p < 0.05$ vs all other groups. ^d $p < 0.005$ vs OLC and surfactant-OLC. ^e $p < 0.005$ vs OLC. ^f $p < 0.05$ vs healthy. ^g $p < 0.05$ vs surfactant-OLC. ^h $p < 0.005$ vs lavaged, OLC and surfactant-OLC. ⁱ $p < 0.05$ vs surfactant and saline. ^j $p < 0.005$ vs healthy. ^k $p < 0.005$ vs surfactant.

Table 4. Data (mean \pm SD) on semi-quantitative histological lung injury scores

	Bacterial infiltration	Cellular infiltration	Edema	Hyaline membranes	Total score
Healthy	0.7 \pm 1.1	4.3 \pm 1.5	0.7 \pm 0.5	0.0 \pm 0.0	5.7 \pm 2.0
Lavaged	11.3 \pm 0.6 ^a	10.7 \pm 1.2 ^d	9.7 \pm 1.2 ^a	4.7 \pm 1.5 ^g	36.3 \pm 1.5 ^a
Surfactant	5.0 \pm 3.0 ^b	7.0 \pm 2.0 ^e	4.0 \pm 2.6	1.7 \pm 1.5	17.6 \pm 9.0 ^b
OLC	4.3 \pm 0.6	5.7 \pm 0.6 ^f	2.0 \pm 1.0	0.0 \pm 0.0	12.0 \pm 1.0 ^b
Surfactant-OLC	5.7 \pm 1.5 ^c	7.7 \pm 1.5 ^e	2.6 \pm 0.6	1.3 \pm 1.5	17.3 \pm 4.6
Saline	0.0 \pm 0.0	1.3 \pm 1.2	0.3 \pm 0.5	0.0 \pm 0.0	1.7 \pm 1.5

Healthy, GBS + PPV_{CON}. *Lavaged*, lavaged + GBS + PPV_{CON}. *Surfactant*, lavaged + GBS + surfactant + PPV_{CON}. *OLC*, lavaged + GBS + PPV_{OLC}. *Surfactant-OLC*, lavaged + GBS + surfactant + PPV_{OLC}. *Saline*, lavaged + saline + PPV_{CON}. ^a $p < 0.01$ vs all other groups. ^b $p < 0.01$ vs lavaged and $p < 0.05$ vs saline. ^c $p < 0.01$ vs lavaged and saline, and $p < 0.05$ vs saline. ^d $p < 0.01$ vs healthy and saline, and $p < 0.05$ vs OLC. ^e $p < 0.01$ vs saline. ^f $p < 0.05$ vs lavaged and saline. ^g $p < 0.01$ vs healthy, OLC and saline, and $p < 0.05$ vs surfactant-OLC.

Discussion

This study demonstrated that a surfactant preparation, consisting mainly of phospholipids, SP-B and SP-C, is able to attenuate bacterial proliferation in the lung and more importantly bacterial translocation to the blood stream. Furthermore, this study suggests that this attenuation is at least in part mediated by a reduction in the degree of atelectasis.

The fact that only the healthy non-lavaged animals in this study were able to clear GBS bacteria from the lung, seems to confirm the importance of local pulmonary host defense factors like endogenous surfactant and AMs [8](#), [11](#). Whole lung lavage, which induces surfactant deficiency and removes part of the local host defense factors like AMs [26](#), [27](#), resulted in GBS proliferation in the lung and bacterial translocation into the blood stream in nearly all animals. These changes also had a severe clinical impact with deteriorating lung mechanics and hemodynamics, resulting in an almost 80% mortality despite the use of intravascular volume expansion and inotropic support. Restoring the surfactant system with exogenous surfactant reduced both GBS proliferation and translocation, and also prevented septic shock and subsequent death in all animals.

To test the hypothesis that this effect of exogenous surfactant was mediated by a reduction in atelectasis, animals were ventilated after lung lavage using an open lung approach, which aims to recruit collapsed alveoli and maintain alveolar patency by applying sufficient PEEP [18](#), [28](#). In the present study we assessed the degree of atelectasis indirectly by measuring arterial oxygenation. Both experimental and human studies have shown an excellent correlation between oxygenation and lung volume [29](#), [30](#). OLC ventilation reduced bacterial translocation comparable to the surfactant treated animals, while GBS proliferation in the lung was even more attenuated.

These results suggest that the attenuation of bacterial growth and translocation by exogenous surfactant is indeed in part mediated by a reduction in atelectasis. The increased reduction in GBS proliferation in the OLC group seems to be consistent with this assumption because, based on oxygenation, the high mean airway pressures during OLC ventilation are more effective in reversing atelectasis than exogenous surfactant.

We can only speculate on the reasons why a reduction in atelectasis mitigated bacterial growth in the lung. First of all, Shennib and colleagues showed that the *in vitro* function of AMs can be impaired if the lung is subjected to several hours of atelectasis [31](#). Second, both the wet-lung weight and the histological evaluation showed that animals subjected to lung lavage had a higher degree of interstitial and alveolar edema compared with

animals treated with exogenous surfactant or OLC ventilation, which might also have impaired antibacterial activity of the AMs [32](#).

Besides atelectasis, other factors might also have played a role in the reduction of bacterial growth after surfactant treatment. First, surfactant treatment has been shown to induce endogenous SP-A production, which might result in increased bacterial clearance [33](#). [34](#). However, we found no differences in SP-A content of the alveolar wash at the end of the ventilation period. Second, *in vitro* experiments have shown that some surfactant preparations are able to mitigate growth of GBS directly [35](#). The present study suggests that this direct inhibitory effect of surfactant on bacteria is of limited importance *in vivo*, because adding surfactant to the OLC group did not further reduce bacterial growth. Third, recent studies have shown that overexpression of SP-B inhibits endotoxin-induced lung inflammation and SP-C interacts with bacterial lipopolysaccharide, indicating a possible role for these hydrophobic surfactant proteins in pulmonary host defense [36](#) [37](#). However, to date it is unknown if these effect are also present *in vivo* when administering these surfactant proteins as part of natural modified surfactant in experimental pneumonia. Future studies need to address these unresolved issues. Finally, *in vitro* experiments have shown that surfactant can suppress the release of different cytokines such as tumor necrosis factor- α from human AMs or monocytes, and it has been suggested that this suppression might be beneficial in *non-bacterial* pulmonary inflammation [38](#) [39](#). However, recent studies in *bacterial* inflammation using both Gram-positive and Gram-negative bacteria to induce experimental pneumonia have reported that proinflammatory cytokines, such as tumor necrosis factor- α , are essential for bacterial clearance from the lung, making this explanation for the reduction in bacterial growth due to surfactant unlikely [40-42](#).

Besides mitigating bacterial growth in the lung, reducing atelectasis by either surfactant treatment or OLC ventilation also resulted in a lower rate of bacterial translocation from the lung into the blood stream. Although the reduced bacterial burden could explain this reduction in bacterial translocation, other mechanisms should be considered. It has been suggested that bacteria present in the lung may enter the blood stream directly through the alveolar epithelial barrier [43](#).

High tidal volumes (volutrauma) and repeated opening and collapse of atelectatic lung units (atelectrauma) during mechanical ventilation, can increase the permeability of the alveolar epithelium [12](#), [44](#) and lead to decompartmentalisation of a *non-bacterial* inflammatory response in the lung [45](#). Reducing atelectasis by either surfactant therapy or high levels of PEEP, attenuates these permeability changes [46-48](#). This might in part explain the reduced

bacterial translocation in both the surfactant and the OLC group. Our findings are consistent with previous reports showing that high levels of PEEP mitigate bacterial translocation in experimental pneumonia [13, 48](#). However, in contrast to these studies, in the present study we ventilated the animals with a low tidal volume. This seems to indicate that even during a low-stretch ventilation strategy, insufficient PEEP resulting in alveolar collapse can lead to increased bacterial translocation.

The most striking finding in this study was the complete reversal of bacterial translocation after adding surfactant to OLC ventilation. This finding suggests an additional effect of surfactant on translocation, not mediated through a reduction in atelectasis. Indeed, animal experiments have shown that surfactant preserves alveolar epithelial permeability independent of the degree of atelectasis or changes in mechanical ventilation [49](#). Furthermore, *in vitro* experiments showed that dipalmitoyl phosphatidylcholine, the major component of human surfactant, attenuates alveolar epithelial injury by GBS hemolysin [50](#).

The present study has several limitations that need to be addressed. First, we cannot rule out that the lavage procedure itself enhanced bacterial translocation, although previous studies have shown that the histological alterations in the lung after a lavage procedure are mild [18, 22](#), as substantiated by the low lung injury score in the *saline* group after 5 hours of ventilation. Second, because of to different degrees of atelectasis, oxygenation also varied between the groups, ranging from normoxia in lavaged animals to hyperoxia in animals treated with exogenous surfactant and/or OLC ventilation.

However, recent data showing that hyperoxia increases rather than decreases bacterial growth and translocation during experimental pneumonia, strengthen rather than weaken the results of the present study [51](#).

Although extrapolation of animal data to humans should be done with caution, we feel that this study might have important implications for the pathogenesis of ventilator-associated pneumonia. In many patients with acute respiratory failure there is evidence for surfactant abnormalities leading to increased alveolar surface tension and subsequent atelectasis [52-54](#). Based on the present study these changes can lead to increased bacterial growth in the lung and, more importantly, increased bacterial translocation into the blood stream leading to severe septic shock. Indeed, patients suffering from acute respiratory distress syndrome have an increased risk of pulmonary infection and often succumb to dissemination of the pulmonary infection with overwhelming sepsis and multiple organ failure [55, 56](#). Our findings also seem to indicate that early surfactant treatment and application of a lung protective ventilation strategy aiming at minimizing both alveolar stretch and collapse might prove

beneficial in reducing the risk for pneumonia in ventilated patients and reduce the incidence of sepsis and mortality often seen in these patients. A recent report in preterm infants with GBS pneumonia showed that exogenous surfactant was well tolerated and improved short-term outcome parameters [57](#).

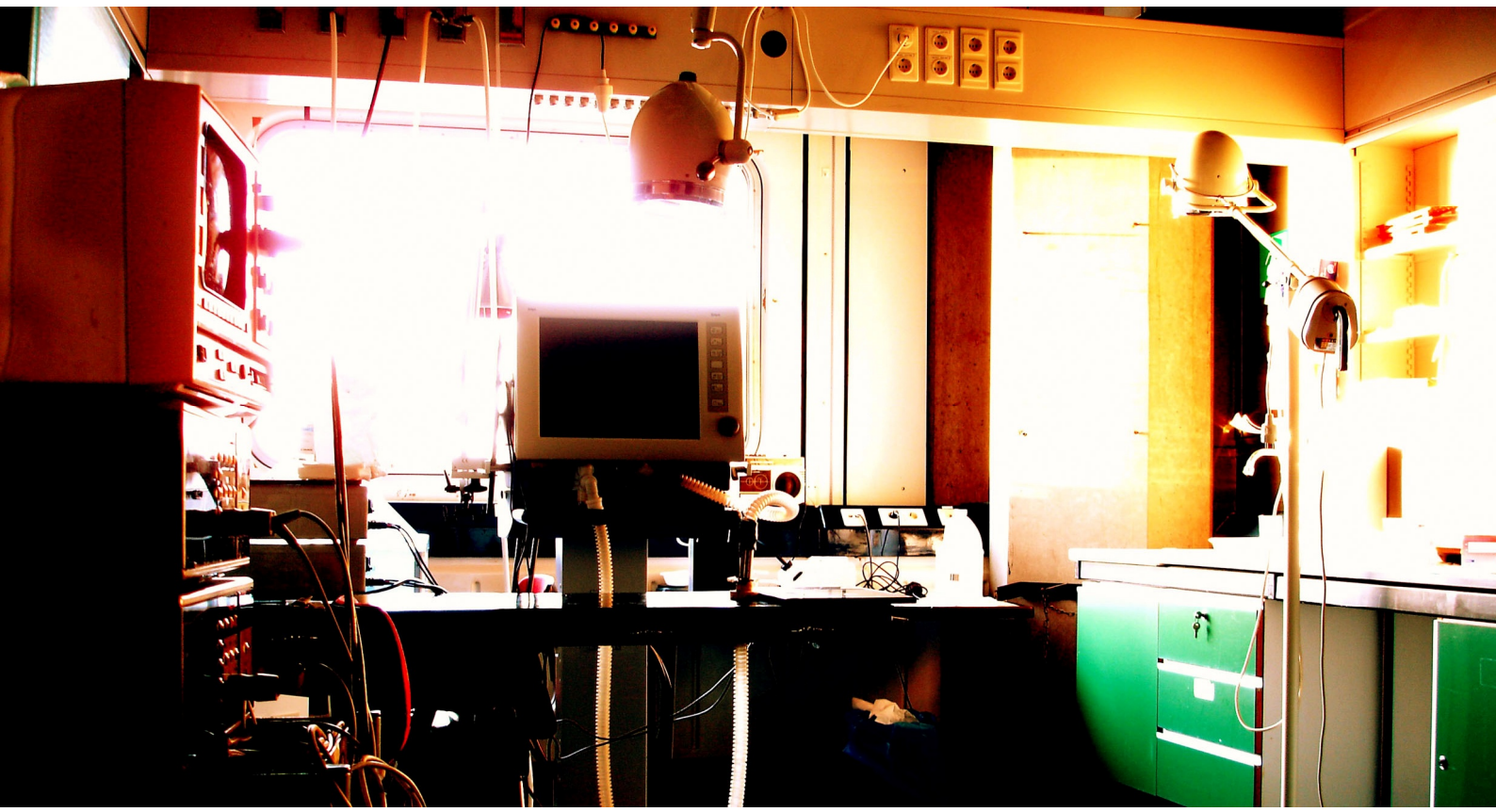
In conclusion, this study shows that natural surfactant mitigates bacterial growth and attenuates bacterial translocation in experimental GBS pneumonia. A reduction in the degree of atelectasis is one of the mechanisms responsible for these beneficial effects. This goal can also be achieved by an open lung ventilation strategy. Our findings offer new insights into the pathogenesis of ventilator-associated pneumonia and subsequent sepsis in patients with acute respiratory failure. In addition, our results emphasize the importance of lung protective ventilation and surfactant therapy in respiratory failure.

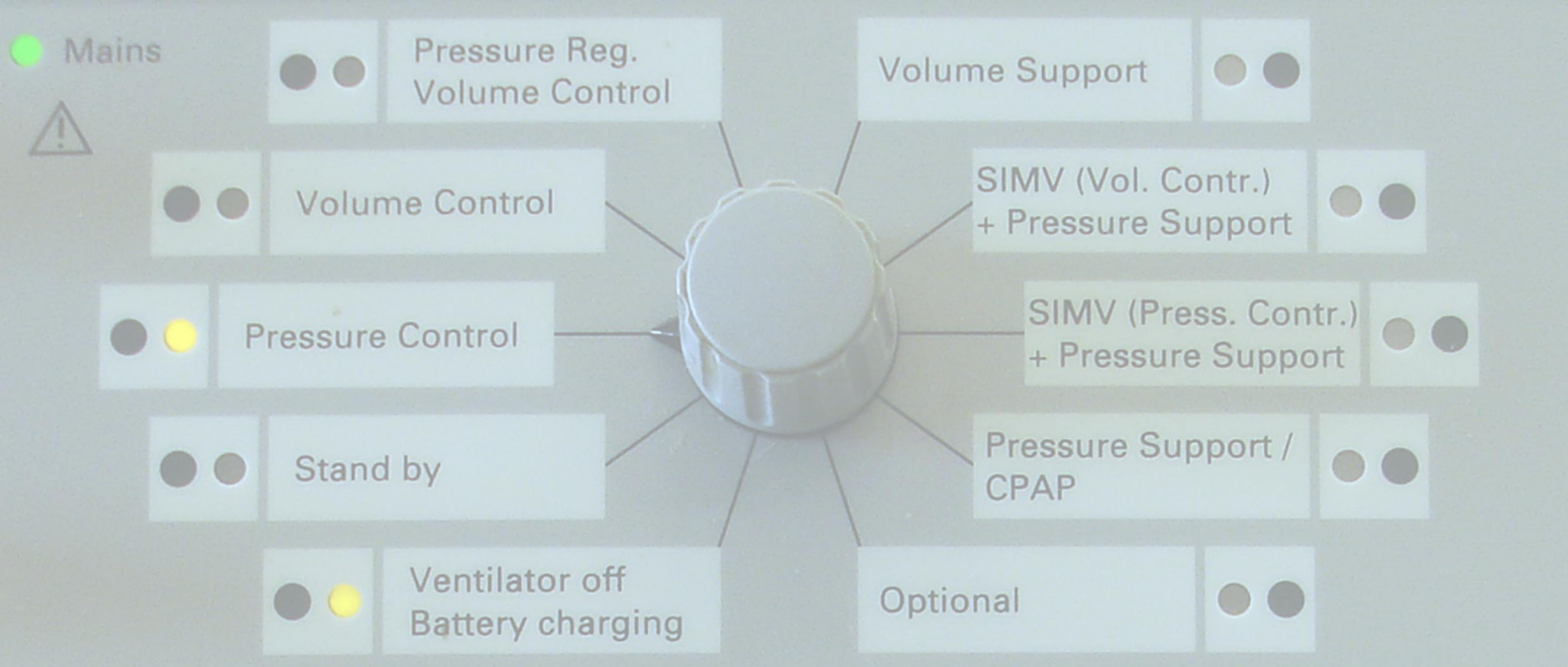
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Resp. pattern

Measured freq.
b/min

50

Set freq.
b/min

50

Insp. period
s

0.40

Insp. flow
l/s

Chapter 6

Positive end-expiratory pressure levels higher than required to prevent atelectasis promote bacterial translocation in an experimental pneumonia model

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Abstract

Objective: A previous study in piglets with an experimental pneumonia has shown that reducing atelectasis by means of Open Lung ventilation attenuated bacterial translocation compared to conventional ventilation settings. In this study, we examined the effect of Open Lung ventilation with higher than necessary positive end-expiratory pressures (PEEP) on bacterial translocation.

Interventions: Thirty piglets were surfactant depleted by whole lung lavage followed by intratracheal instillation of $\sim 10^{10}$ group B streptococci. Thereafter, the animals were ventilated for 5 h according to either a conventional ventilation strategy, Open Lung strategy or Open Lung high PEEP strategy. Blood samples for blood gas analysis and blood bacterial counts were taken every hour. After 5 h of ventilation, surviving animals were killed and lung colony forming units and lung mechanics parameters were determined.

Results: There was a mortality of 70% in the conventional ventilation group while all animals in both Open Lung groups survived the 5 h ventilation period. Open Lung ventilation resulted in significantly less bacterial translocation (time to bacteremia and absolute bacterial counts in blood) compared to both conventional and high PEEP ventilation, the latter two having comparable translocation rates.

Conclusions: The present study suggests that optimal ventilation can only be achieved by using individual settings that may differ between individual patients (Open Lung concept). Deviation to either side can be harmful, as shown by the increased bacterial translocation during conventional and high PEEP ventilation.

Introduction

Patients on intensive care units with an endotracheal tube are often colonized with potential pathogenic micro-organisms which eventually might lead to the development of pneumonia [1-3](#). Sepsis and/or septic shock complicate the clinical picture [4-6](#). At the same time it is not uncommon that the underlying pneumonia may be the source for the sepsis-related bacteremia [7](#). While the ventilation procedure itself can be responsible for the spillover of bacteria into the systemic circulation [8, 9](#), little is known about the exact mechanism of this potentially disastrous process. The few experimental studies that have investigated this issue indicate that injurious ventilation with high tidal volumes using little or no positive end-expiratory pressure (PEEP) facilitate bacterial translocation while the addition of higher PEEP values could attenuate it [8, 9](#). However, setting the right level of PEEP in critically ill patients with ARDS remains a highly controversial point of debate [10](#). PEEP amounts suggested in literature range from as low as possible to achieve acceptable oxygenation [11-13](#) to very high values of around 35 cmH₂O [14](#). Another concept of finding the right PEEP was introduced by Lachmann in 1992 [15](#) by propagating the notion of giving the patient not one universal best amount of PEEP but rather only the PEEP that the individual actual needs for an open lung (characterized by a PaO₂/FiO₂ ratio above 400). Using this “Open Lung Concept” van Kaam and colleagues could show that bacterial translocation was attenuated in an experimental pneumonia model as compared to conventional ventilation with PEEP 5 cmH₂O and a tidal volume of 7 ml/kg [16](#). However, applying this concept properly also implies constant re-evaluation of the individual ventilatory settings, often a difficult task in the hectic ICU environment. But ignoring the latter by, for instance, not adjusting PEEP levels when lung function improves or by using a universal high PEEP for all patients, may eventually result in ventilating the individual with pressures higher than necessary and thus causing over distension. Although it has been stated elsewhere that high end-expiratory pressures are not injurious to the lung [17](#), to our knowledge, nothing is known about the influence of high PEEP ventilation on possible pulmonary bacterial translocation. To shed more light into this unknown territory, we used the same experimental model as van Kaam and colleagues [16](#) but ventilated the animals with a higher PEEP than required for optimal gas exchange, and compared the number of blood colony forming units (CFUs) as the main outcome variable to conventional and “Open Lung Concept” ventilation.

Material and Methods

Animals

The institutional Animal Investigation Committee Care approved the study protocol and handling of the animals was in accordance with the latest European Community guidelines (86/609/EC).

Bacteria

Stationary-phase cultures were prepared by incubation of group B streptococci (GBS) aliquots for 16 h at 37°C in 12 ml trypticase soy broth (TSB 3%, Becton Dickinson, Cockeysville, MD, USA). Thereafter, 8 ml of this broth was taken and combined with 50 ml fresh TSB and incubated at 37°C for 3 h. The bacteria were then centrifuged, washed and resuspended in sterile saline at a concentration of approximately 10^8 CFU per ml as determined by spectrophotometric measurement of optical density (OD) at 595 nm. To verify the number of viable bacteria in the inoculum, 100 μ l of 10-fold dilution steps in saline were plated on blood agar plates and incubated overnight at 37°C. CFUs were visually counted the following day.

Animal preparation

Anesthesia was induced in 30 newborn piglets (aged 77 h \pm 15 (SD) with ketamine hydrochloride (35 mg/kg, i.m.) and midazolam (0.5 mg/kg, i.m.). The animals were tracheotomized and thereafter ventilated in the pressure controlled time-cycled mode (Servo 300 Maquet, Solna, Sweden) at a fractional inspired oxygen concentration of 1.0, PEEP of 2 cmH₂O, a rate of 30 breaths/minute and an inspiratory/expiratory (I/E) ratio of 1:2. Tidal volume was measured at the Y-piece using a stand-alone respiratory monitor (CO₂SMO Plus, Novamatrix Systems, Wallingford, CT, USA). Peak inspiratory pressures (PIP) were adjusted to keep the expiratory tidal volumes around 7 ml/kg. A neuromuscular block was induced with pancuronium bromide (0.5 mg/kg, i.v.), followed by a continuous infusion of fentanyl (20 μ g/kg/h), midazolam (0.3 mg/kg/h) and pancuronium bromide (0.3 mg/kg/h).

Using an aseptic technique, catheters were inserted into the external jugular vein for measurement of central venous pressure and infusion of fluids and medication, and the carotid artery for monitoring of blood pressure and blood sampling.

A continuous infusion of 5% dextrose was started (100 ml/kg/d) and body temperature was kept between 38° and 39°C during the experiments.

Lavage procedure

Respiratory failure was induced by removing the endogenous surfactant through repeated saline lavage (50 ml/kg; 37°C) as previously described ¹⁸. Lavages were repeated at 3-minute intervals until PaO₂ was below 80 mmHg and PaCO₂ above 40 mmHg at the following ventilator settings: PIP/PEEP 20/5 cmH₂O, rate 30 breaths/minute, I/E ratio 1:2 and a fractional inspired oxygen concentration of 1.0. The time needed for the lavage procedure was approximately 45 minutes.

GBS instillation

After lavage, the animals received 10 ml/kg of the GBS suspension. This suspension was slowly injected through a catheter placed at the end of the endotracheal tube. To ensure equal distribution, the first 5 ml/kg were injected with the animal placed on its right side. After 5 minutes of ventilation the animal was turned to the left side and a second dose of 5 ml/kg was injected in a similar fashion. This bacterial instillation procedure is in accordance with our previous study that showed similar bacterial viability between the GBS solution and GBS bacteria in the lung 5 minutes after intratracheal injection ¹⁶. Furthermore, this instillation technique produced an excellent correlation between the number of CFU/lung calculated on the basis of the left lung and both the left and the right lung, indicating an even distribution of the GBS solution between the right and left lung after intratracheal injection ¹⁶. Following GBS instillation the animals were returned to the supine position for the remainder of the experiments.

Ventilation strategies and experimental groups

All animals received positive pressure ventilation, but different ventilation strategies depending on their group. Fractional inspired oxygen concentration was kept at 1.0 during all experiments. All groups consisted of 10 animals, the ventilation period after GBS installation was 5 h.

Conventional positive pressure ventilation – group ConV (10 piglets)

The animals of the ConV group were ventilated in the pressure controlled mode. The PIP was set at a level that resulted in an expiratory tidal volume of approximately 7 ml/kg. The level

of PEEP was maximized at 5 cmH₂O and the ventilatory rate was set to 60 breaths/min (I/E ratio of 1:2).

Open lung concept positive pressure ventilation – group OLC (10 piglets)

As previously described [16](#), the main objective of this ventilation strategy is to recruit atelectatic lung regions using high levels of PIP for a short period of time and to prevent repeated alveolar collapse by applying sufficient levels of PEEP [18](#). In addition, pressure amplitudes (PIP minus PEEP) were minimized as much as possible in order to prevent alveolar overdistension, and hypercapnia was prevented by using supranormal ventilatory rates (I/E ratio of 1:1, 100 breaths/minute).

Changes in oxygenation and subsequent changes in intrapulmonary shunt were used to assess alveolar collapse. For this reason, a sensor for continuous blood gas monitoring (Paratrend, Diametrics Medical Ltd., Buckinghamshire, UK) was inserted through a femoral artery catheter. Based on PaO₂ levels in the healthy piglets ventilated with a fractional inspired oxygen concentration of 1.0, we defined optimal alveolar recruitment when PaO₂ ≥ 450 mmHg.

High PEEP positive pressure ventilation – group High PEEP (10 piglets)

After recruitment (see *OLC group*), PEEP was set 15 cmH₂O, 5 cmH₂O higher than the optimal PEEP determined in our previous study for the Open Lung animals (10 cmH₂O) [16](#). This PEEP level was chosen based on preliminary experiments that showed that PEEP levels higher than 15 cmH₂O were not well tolerated hemodynamically. To prevent hypercapnia, peak pressures were set to yield a tidal volume of 6-7 ml/kg with ventilatory rates in the supranormal range (I/E ratio of 1:1, 100 breaths/minute).

Hemodynamic support

Hemodynamic deterioration was defined as: 1) a decrease mean arterial blood pressure of more than 10%, and 2) a heart rate > 200 beats/minute or an increase of more than 10% if baseline values were already above this threshold value. We used the mean arterial blood pressure and heart rate during the first hour of ventilation after GBS instillation as baseline values. If both criteria were met, intravascular volume expansion was administered, using sterile saline with a maximum cumulative dose of 50 ml/kg. In addition, dopamine infusion was started after administration of the first 20 ml/kg saline, in a dose of 20 µg/kg/min.

Data acquisition and outcome parameters

Ventilation and hemodynamics

Ventilatory parameters (airway pressures, tidal volumes, ventilatory rates) and hemodynamic parameters (mean arterial blood pressure, central venous pressure, heart rate) were recorded after the instrumentation period, after lung lavage, and every 15 minutes after GBS instillation.

Blood gas analysis

Samples for blood gas analysis (ABL 555, Radiometer, Copenhagen, Denmark) were drawn at the end of the instrumentation period, after lung lavage, and hourly after GBS instillation. To evaluate possible negative effects of high ventilation pressures on hemodynamics and blood gases, ventilation pressures in the High PEEP group were reduced to average OLC values (PIP: 17 cmH₂O, PEEP: 10 cmH₂O - based on our previous study [16](#)) at the end of the 5 h ventilation period. In the High PEEP group a final blood sample for gas analysis was drawn 5 min after this procedure (5 h'5).

CFU in blood

Blood cultures were drawn under aseptic conditions at the end of the instrumentation period, just prior to the instillation of the GBS solution, and hourly thereafter. The number of CFU/ml blood was calculated by spreading 1 ml of whole blood on a blood agar plate (Becton Dickinson, Alphen a/d Rijn, the Netherlands), which was incubated for 24 hours at 37°C.

Survival

Survival time starting after GBS instillation was recorded for all animals. Those animals still alive after the 5 h ventilation period were killed using an overdose of pentobarbital.

Lung function

The thorax and diaphragm was opened immediately after death and pressure-volume (P/V) curves were constructed using the syringe technique, as previously described [18](#). Maximal lung compliance was calculated from the steepest slope on the deflation limb. Lung volumes at transpulmonary pressures of 35 (total lung capacity) and 5 cmH₂O were recorded from the deflation limb.

CFU in lung homogenate

After clamping the left main bronchus, the left lung was dissected, weighed and after adding 25 ml sterile saline, homogenized at 4°C for 2 minutes at 40,000 rpm in a tissue homogenizer (Virtis “23”, The Virtis Company Inc., NY)⁸. The volume of the lung homogenate was recorded and a 1 ml aliquot was serially diluted and spread on blood agar plates for calculation of the number of viable CFU/ml homogenate. Using the data on homogenate volume, left lung weight and total lung weight, the number of CFU/lung was calculated.

Broncho-alveolar lavage (BAL)

After dissecting the left lung, BAL of the right lung was performed five times (40 ml/kg) with a saline solution supplemented with 1.2 mM CaCl₂. The fluids recovered from these five lavages were pooled and analyzed as one sample. The percentage of lung lavage fluid recovered was calculated. Samples were centrifuged for 10 minutes at 1500 g to remove cell material. Protein concentration was measured using the Bradford method (Biorad protein assay, Munich, Germany)¹⁹.

Statistical analysis

All data are expressed as mean \pm SD. Data on bacterial growth were subjected to logarithmic transformation (\log_{10}). Intergroup differences were analyzed with the analysis of variance followed by a Bonferroni post-hoc test. The Kaplan Meier analysis followed by a log rank test was used to compare survival and bacterial translocation. Intergroup absolute numbers of blood CFUs were compared using the Kruskal-Wallis test. A Fisher's exact test was used when appropriate. Data from the ConV group at 5 h (n=3) were excluded from statistical analysis. A *p*-value of 0.05 or less was considered statistically significant.

Results

Animals

The mean weight and age of the studied animals was 1.9 ± 0.4 kg and 77 ± 15 hours, respectively. There were no intergroup differences in age, weight and number of lavages needed to induce lung injury. No air leaks were observed in the animals during the study period.

Survival

Seven of the 10 animals in the ConV group died during the ventilation period, with a mean survival time of 258 ± 13 minutes. This was significantly different compared to the 100% survival of the remaining groups.

CFUs in lung homogenate

The number of CFUs instilled intratracheally was similar in the three groups (Figure 1).

After 5 h of ventilation the number of CFU/lung increased significantly in the ConV group and was also significantly higher compared to the OLC and High PEEP groups. There was neither bacterial growth nor clearance in the latter two groups.

The total lung weight corrected for body weight at the end of the experiments was significantly higher in the ConV group compared to the OLC and High PEEP groups (Table 1).

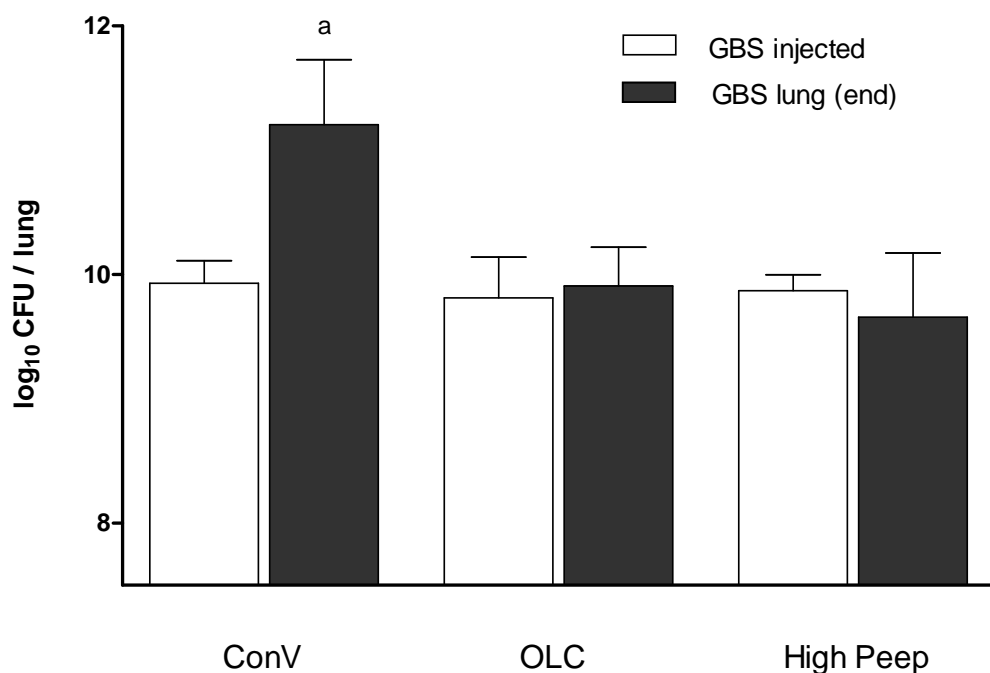


Figure 1. The initial number of CFUs (mean \pm SD) expressed as log₁₀ CFU per lung, injected in the lung and the subsequent proliferation during the ventilation period. ConV = lavaged + GBS + conventional ventilation; OLC = lavaged + GBS + Open Lung ventilation; High PEEP = lavaged + GBS + high PEEP ventilation. ^ap < 0.001 vs. GBS injected, OLC and High PEEP

Table 1. Data on lung weight, lung mechanics and alveolar protein influx

	ConV	OLC	High PEEP
WW/BW, g/Kg	49 ± 8*	37 ± 3	34 ± 6
C _{Lmax} , ml/cm H ₂ O/kg	1.3 ± 0.7*	5.0 ± 1.6	5.8 ± 1.6
TLC ₃₅ , ml/kg	24 ± 8*	59 ± 15	69 ± 14
V ₅ ml/kg	11 ± 6*	39 ± 11 [§]	51 ± 14
Protein, mg/ml	1.39 ± 0.54*	0.79 ± 0.19	0.8 ± 0.18

Definition of abbreviations: WW/Bw = wet weight lung weight to body weight ratio; C_{Lmax} = maximal lung compliance; TLC₃₅ = lung volume at a transpulmonary pressure of 35 cm H₂O (total lung capacity); V₅ = lung volume at a transpulmonary pressure of 5 cm H₂O.

Data presented are mean ± SD. ConV = lavaged + GBS + conventional ventilation; OLC = lavaged + GBS + Open Lung ventilation; High PEEP = lavaged + GBS + high PEEP ventilation, *p < 0.05 vs. OLC and High PEEP, [§]p < 0.05 vs. High PEEP

CFUs in blood

The animals were blood negative for GBS prior to bacterial instillation. As shown in Figure 2, all but one animal in the ConV group had GBS positive blood cultures, with a mean time to bacteremia of 102 ± 23 minutes. All animals of the High PEEP group had positive blood cultures at the end of the ventilation period with a mean time to bacteremia of 83 ± 16 minutes.

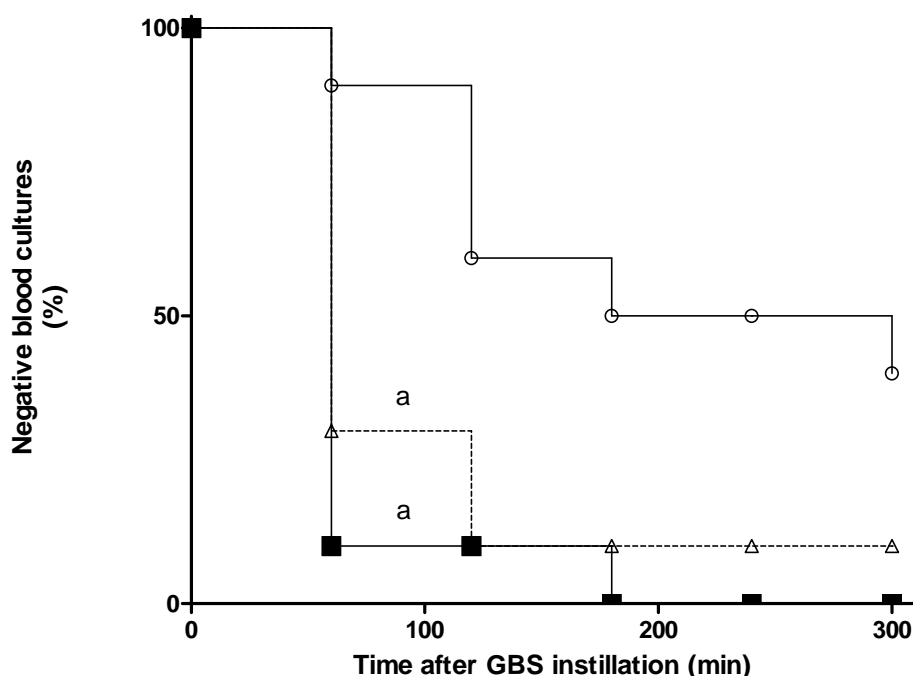


Figure 2. Kaplan-Meier curves displaying the percentage of animals in each group with negative blood cultures during the 5 h ventilation period. ConV = lavaged + GBS + conventional ventilation (triangles); OLC = lavaged + GBS + Open Lung ventilation (circles); High PEEP = lavaged + GBS + high PEEP ventilation (squares). ^ap < 0.01 vs. OLC

The use of OLC ventilation resulted in a significant increase in time to bacteremia (210 ± 33 min) with 6 of the 10 animals being GBS blood positive after 5 h of ventilation. The absolute number of CFUs per millilitre blood was significantly lower in the OLC group compared to the other two groups from 2 h onwards (Figure 3).

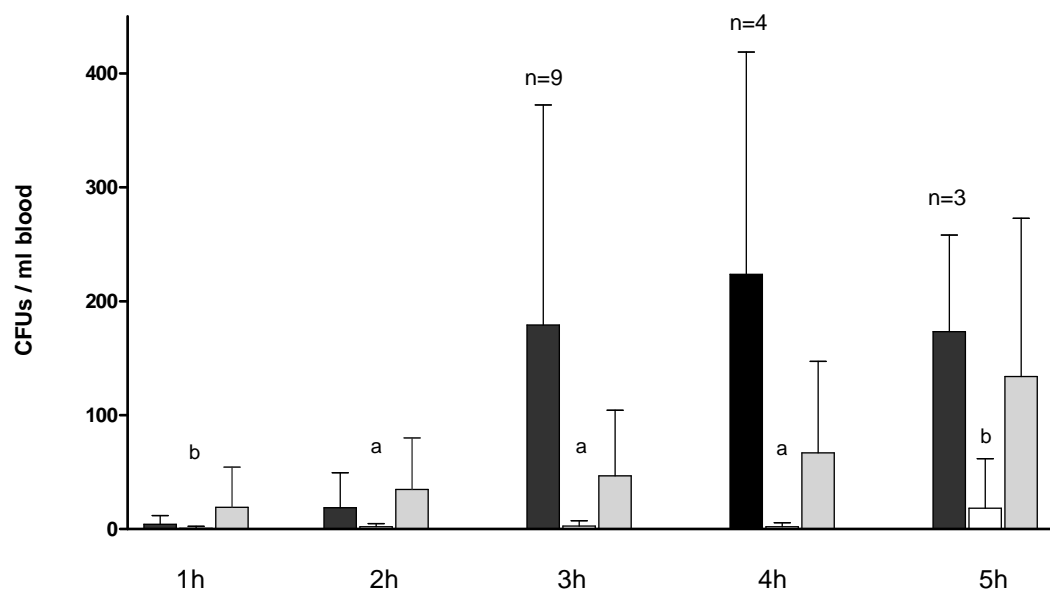


Figure 3. Blood CFUs over time (mean \pm SD). ConV = lavaged + GBS + conventional ventilation (black bars); OLC = lavaged + GBS + Open Lung ventilation (white bars); High PEEP = lavaged + GBS + high PEEP ventilation (grey bars). ^a $p < 0.05$ vs. ConV and High PEEP, ^b $p < 0.05$ vs. High PEEP

Gas Exchange

PaO_2 and PaCO_2 levels after the instrumentation period and after lung lavage were comparable in the three groups (Figure 4). In the ConV group, oxygenation was severely impaired and did not improve from post lavage values throughout the 5 h ventilation period (Figure 4A). Ventilation according to the OLC significantly improved oxygenation to healthy baseline levels for the remainder of the experiment. The animals of the High PEEP group also showed improved oxygenation but significantly less compared to the OLC group. Reducing the ventilatory pressures in these animals to OLC levels at the end of the experiment resulted in a significant increase in oxygenation to healthy baseline values (Figure 4A).

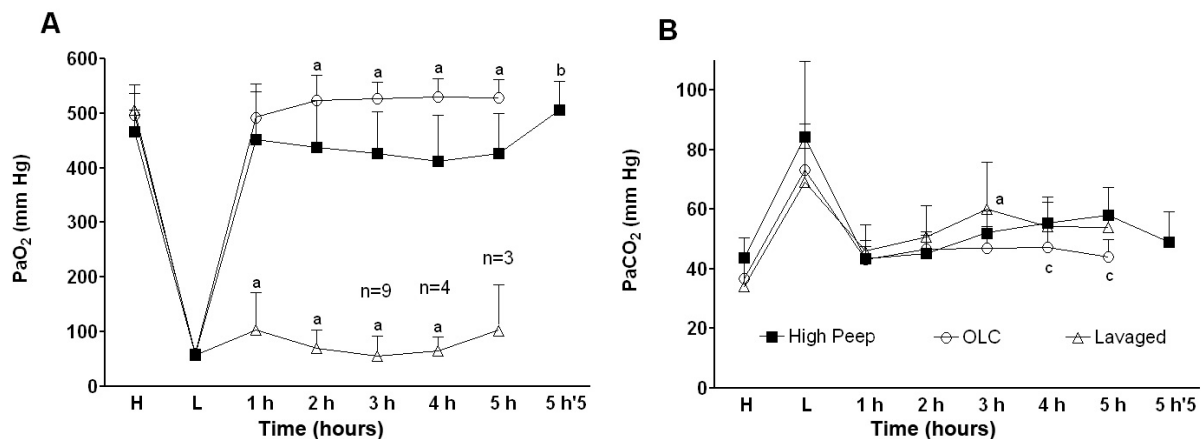


Figure 4. Changes (mean \pm SD) in PaO₂ (A) and PaCO₂ (B) levels in the three groups.

H = healthy baseline value, L = after lavage; ConV = lavaged + GBS + conventional ventilation (triangles); OLC = lavaged + GBS + Open Lung ventilation (circles); High PEEP = lavaged + GBS + high PEEP ventilation (squares); 5 h'5 = open lung concept ventilatory settings, measured 5 min after ventilation settings were set to PIP 17 cmH₂O and PEEP 10 cmH₂O after 5 h of ventilation. ^ap < 0.05 vs. the two other groups, ^bp < 0.05 vs. High PEEP 1 h, 2 h, 3 h, 4 h and 5 h, ^cp < 0.05 vs. High PEEP

Ventilatory and Circulatory Parameters

There were no differences in ventilatory and circulatory parameters between the three groups before and immediately after lung lavage. The mean end-expiratory tidal volume was slightly below 7 ml/kg in the High PEEP and OLC groups and within this range in the ConV group (Table 2). In all animals, expiratory flow was observed to be zero prior to each inspiration indicating the absence of intrinsic PEEP.

In contrast with the other groups, the ConV group showed a deteriorating mean arterial blood pressure over time, which was accompanied by an increase in heart rate (Table 3). Mean arterial pressure was stable but lower in the High PEEP group compared to the OLC group (Table 3). In accordance with these findings, all 10 animals in the ConV group compared to 1 in the OLC group and 4 in the High PEEP group required volume support ($p < 0.05$). Similarly, all but one animal in the ConV group but no animal in the other groups required dopamine infusion ($p < 0.05$).

Pressure-Volume Curves

P/V curves recorded postmortem showed a severe deterioration of lung function in the ConV group (Table 1). Compared to the OLC group, ventilation with high PEEP increased the lung volume at a pressure of 5 cmH₂O on the deflation limb of the P/V curve (Table 1).

Table 2. Ventilatory parameters over time in the three groups

	ConV	OLC	High PEEP
MawP, cm H₂O			
1 h	11.2 ± 1.5*	14.8 ± 1.4 [§]	20.4 ± 0.7
2 h	12.2 ± 1.9*	14.9 ± 1.2 [§]	20 ± 0.7
3 h	10.3 ± 0.6	13.5 ± 1.7 [§]	20.8 ± 0.7 [†]
5 h '5			13.3 ± 0.5
PEEP, cm H₂O			
1 h	4.5 ± 0.7*	10.9 ± 0.9 [§]	15 ± 0.0
2 h	4.9 ± 1.0*	10.8 ± 0.9 [§]	15 ± 0.0
3 h	4.0 ± 0.0	9.1 ± 1.5 [§]	15 ± 0.0 [†]
5 h '5			10 ± 0.0
PA, cm H₂O			
1 h	19.5 ± 3.0*	7.2 ± 1.1 [§]	10 ± 0.8
2 h	21.6 ± 4.25*	8.1 ± 0.7	9.4 ± 1.1
3 h	18.0 ± 1.7	8.8 ± 0.8 ^{§,†}	11 ± 1.0 [†]
5 h '5			7 ± 0.0
VTEX, cm H₂O			
1 h	7.3 ± 0.8*	6.0 ± 0.3	6.2 ± 0.3
2 h	6.6 ± 0.7	6.0 ± 0.4	6.1 ± 0.1
3 h	7.3 ± 1.2	6.3 ± 0.4 [†]	6.0 ± 0.3 [†]
5 h '5			7.2 ± 1.1

Definition of abbreviations: MawP = mean airway pressure; PEEP = positive end-expiratory pressure; PA = pressure amplitude; V_{TEX} = expiratory tidal volume; 5 h'5 = open lung concept ventilatory settings measurement 5 min after ventilation settings were set to PIP: 17 cmH₂O, PEEP: 10 cmH₂O after 5 h of ventilation. Data presented are mean ± SD. For definitions of group classifications see Table 1.

* p < 0.01 vs. OLC and High PEEP, [§]p < 0.01 vs. High PEEP, [†]p < 0.01 vs. High PEEP 5 h'5

Proteins in BAL

Alveolar protein influx was most severe in the ConV group. There was no difference in BAL protein content between the High PEEP and OLC group (Table 1).

Table 3. Circulatory parameters over time in the three groups

	ConV	OLC	High PEEP
MAP, mm Hg			
1 h	68 ± 6	75 ± 8	56 ± 6**
2 h	56 ± 16*	71 ± 7	59 ± 6*
3 h	55 ± 20	67 ± 9	53 ± 7*
5 h '5			54 ± 7
CVP, mm Hg			
1 h	6.6 ± 1.1*	8.6 ± 0.8	7.4 ± 1.6
2 h	6.7 ± 1.5	7.7 ± 0.7	7.2 ± 1.9
3 h	5.8 ± 0.6	7.6 ± 0.7	6.9 ± 1.0
5 h '5			7.0 ± 0.8
HR, beats/min			
1 h	189 ± 35	215 ± 18	177 ± 34*
2 h	219 ± 35	228 ± 34	212 ± 35
3 h	250 ± 43	249 ± 23	219 ± 32*
5 h '5			217 ± 31

Definition of abbreviations: MAP = mean arterial pressure; CVP = central venous pressure; HR = heart rate; 5 h '5 = open lung concept ventilatory settings, measurement 5 min after ventilation settings were set to PIP: 17 cmH₂O, PEEP: 10 cmH₂O after 5 h of ventilation. Data presented are mean ± SD
For definitions of group classifications see Table 1. *p < 0.05 vs. OLC, **p < 0.05 vs. OLC and lavage

Discussion

In the present study we demonstrate that Open Lung ventilation with end-expiratory pressures higher than required for optimal gas exchange promotes bacterial translocation in terms of time to bacteremia and absolute numbers of CFUs found in arterial blood. At the same time, growth of bacteria in lungs remains similar compared to Open Lung ventilation with optimal PEEP.

In a previous study we showed that bacterial growth and translocation could be attenuated by reducing atelectasis in an ARDS model of experimental pneumonia [16](#). It was concluded that using the “Open Lung” concept resulted in less volutrauma and atelectrauma and therefore in less permeability disturbances with subsequently less bacterial translocation [16](#). Although it has been suggested that bacteria present in the lung may enter the pulmonary blood stream directly through the alveolar epithelial barrier [20](#), the precise mechanism still remain elusive. It has been shown that atelectrauma (repeated opening and collapse of

atelectatic lung units) induced through injurious ventilation with little or no PEEP is characterized by the loss of alveolar barrier function [21](#). Therefore, it is plausible that the lung bacteria in the atelectrauma prone ConV group entered the pulmonary blood stream through the disrupted alveolar-capillary membrane. Open lung ventilation (OLC group) on the other hand mitigated atelectrauma and thus attenuated bacterial translocation. Surprisingly, increasing the PEEP from 10 cmH₂O (OLC group) to 15 cmH₂O (High PEEP group) resulted in bacterial translocation rates (time until occurrence and absolute numbers of CFUs) as severe as in the ConV group. However, an increase of atelectrauma cannot be the reason for this increase in translocation because PaO₂ values and lung mechanic parameters - being comparable to those of the OLC group - indicate that the lungs of the High PEEP animals were “open” during the 5 h ventilation period. This finding therefore suggests a second mechanism for the spillover of bacteria from the lung compartment to the systemic circulation. We can only speculate on how the higher PEEP may have caused the augmented translocation. Lung mechanics analysis at the end of the experiment revealed higher values for C_{lmax}, TLC₃₅ and V₅ in the animals of the High PEEP group compared to the OLC group, and compared to the healthy animal group reported in the study by van Kaam and colleagues [16](#). Consequently, in the present study the lungs of the High PEEP group may have been subjected to an increased stretch on the alveolar-capillary membrane (caused by the higher end-expiratory and end-inspiratory lung volume) over distending the lung tissue. To prevent serious hypercapnia in the High PEEP group, peak pressures could not be set to the 18 cmH₂O used in the OLC group but had to be raised to approximately 25 cmH₂O to yield a tidal volume of about 6 ml/kg. However, the resulting pressure amplitude was higher compared to the OLC group (significant at 1 h and 3 h) at similar tidal volumes indicating that ventilation - in comparison to OLC ventilation - took place still further to the right on the flat part of the P/V curve. And, although this overdistension did not lead to a serious compromise of oxygenation, or to edema formation with protein leakage and thus inferior lung compliance, our findings indicate that this increased stretch may have been a factor for the increased bacterial translocation. Studies have shown that progressively stretched epithelium irreversibly opens water-filled channels allowing free diffusion of small solutes and even albumin [22-24](#) but only at inflation in the supra-physiological range [24](#). Also, experimental studies with small solutes like ^{99m}Tc-DTPA have shown that the rate of clearance of this tracer from the alveolar space increases with increases in lung volume, whether caused by large tidal volume ventilation [25](#) or PEEP [26](#). Finally, Dreyfuss and colleagues have shown that the end-inspiratory volume is probably the main determinant of ventilation-induced edema

regardless of whether it is generated by large tidal volumes with low PEEP or small tidal volumes and high PEEP ²⁷. Clearly, the adjustment in peak pressure in our experimental setting makes it difficult to differentiate between the contributing effects of either the higher end-expiratory volume (high PEEP) and/or the higher end-inspiratory volume (high PIP) on bacterial translocation. But it seems plausible that overstretching an already “open lung” with still higher volumes (high PEEP coupled with a higher PIP to ensure an adequate tidal volume for CO₂ elimination) causes significant overstretch to the lung tissue that could have allowed bacteria to traverse the alveolar-capillary membrane. However, inflating the lungs with high peak pressures for only a short consecutive time (45 cmH₂O for 30 seconds) does not seem to increase bacterial translocation ²⁸ suggesting a possible time dependency before measurable translocation occurs. More studies are needed to further elucidate the effect of high lung volumes on bacterial translocation.

Interestingly, the profound bacteremia in the High PEEP group did not have an influence on mortality in contrast to the mere 30% survival in the ConV group. Having the high blood CFU counts in mind, the 100% survival of the High PEEP animals seem to suggest that mortality in this experimental model is due to a combination of both detrimental lung function and bacteremia as opposed to either one of these factors alone. This suggestion is supported by van Kaam and colleagues who showed that mortality is not increased when lung lavaged piglets without pneumonia are ventilated conventionally ¹⁸.

It is known that the use of high PEEP may result in a decrease of venous return, cardiac output and oxygen delivery ²⁹⁻³² by transmitting the higher mean airway pressures to the intrathoracic vessels, thereby reducing the blood flow from the periphery and the cardiac output. This can explain the lower mean arterial blood pressure we observed in the animals of the High PEEP group compared to the OLC group. Even though reducing the PIP and PEEP in the High PEEP animals to levels applied in the OLC group had no immediate effect on mean arterial blood pressure, it did result in an immediate increase of the PaO₂. We speculate that this increase in arterial oxygenation is due to a deceleration of blood flow through the pulmonary capillary that took place after the ventilation pressures were reduced (also reducing the intrathoracic pressure) giving the blood more time to load oxygen. The increase of PaO₂ after reducing the ventilation pressures in the High PEEP group therefore demonstrates the negative influence of high intrathoracic pressures on hemodynamics and also rules out the possibility of atelectasis formation being responsible for the initial lower PaO₂ in the High PEEP animals compared to the OLC group.

Conclusions

Although animal experiments cannot be translated directly into a clinical setting, we still think that this study may have important clinical implications. In recent years there has been growing awareness concerning the issues of ventilator-induced lung injury leading to the general acceptance of avoiding high tidal volumes and therefore volutrauma. But the question on how to ventilate ARDS patients optimally remains and is still a point of controversial debate [10](#), [33](#), [34](#). In accordance with previous studies [16](#), [35](#) the present study concludes that optimal ventilation can only be achieved by using individual settings that may differ from patient to patient. Deviation from these settings (to either side) can be harmful, as shown by the increased bacterial translocation in the ConV and High PEEP groups. The latter becomes especially relevant considering the current high prevalence of ventilator-associated pneumonia in intensive care units and the possible severe consequences bacterial translocation into the systemic circulation could have.

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17

Pause

End exp. 13

100
90
80

Pressure Control Level above PEEP



Pressure Support Level above PEEP

Resp. pattern

CMV freq. b/min

Measured freq. b/min

51

Set freq. b/min

51

Insp. period s

0.33

Insp. flow l/s



Insp. time %



Pause time %

Volume

Volume

Tidal vol. ml

Minute vol. l/min

Insp. tidal vol. ml

11

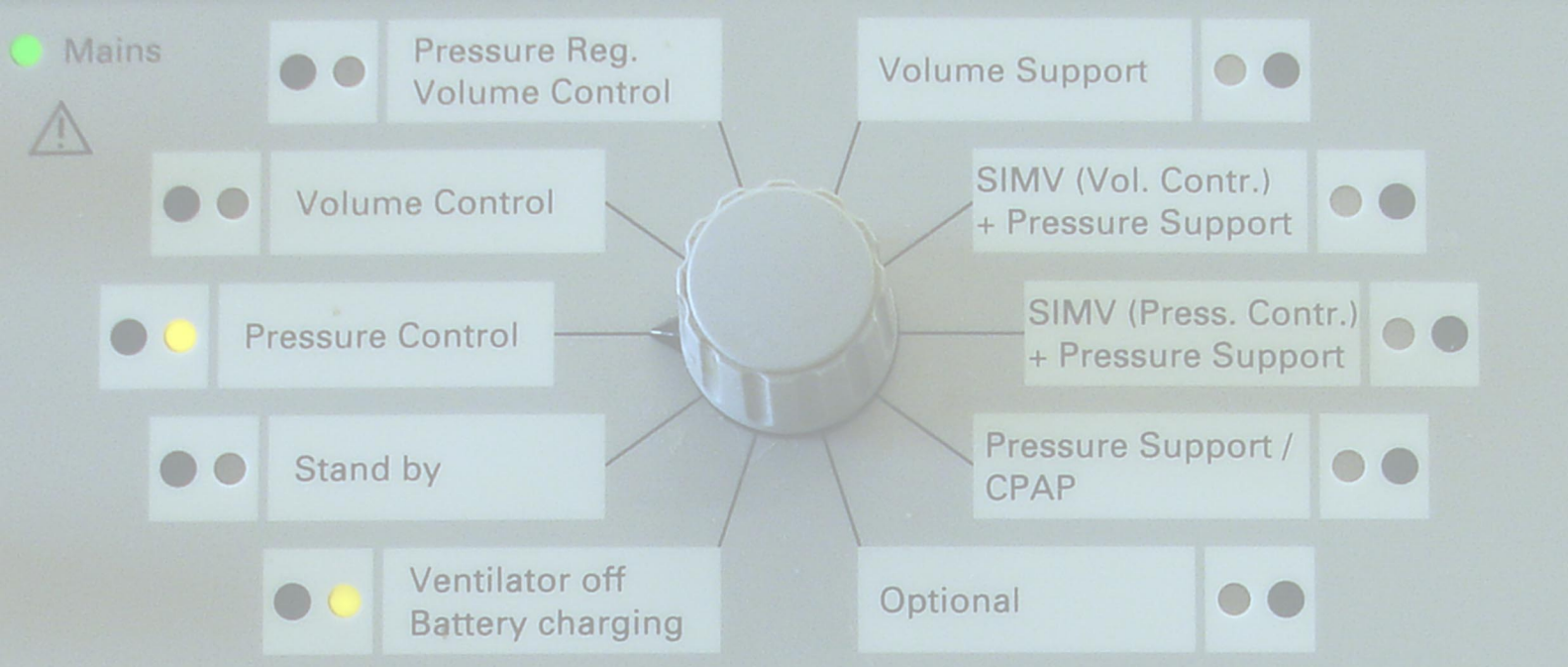
Exp. tidal vol. ml

8



Alarms and messages 02 95

- Airway pressure
- O₂ concentration
- CO₂ concentration
- Exp. minute volume
- Apnea



Resp. pattern

Measured freq.
b/min
50

Set freq.
b/min
50

Insp. period
s
0.40

Insp. flow
l/s

Chapter 7

Immunoglobulin M-enriched intravenous polyclonal immunoglobulins reduce bacteremia following *Klebsiella pneumoniae* infection in an ARDS rat model

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Abstract

Mechanical ventilation is known to induce bacterial translocation from the lung into the systemic circulation. This study determined the effect of IgM-enriched polyclonal immunoglobulins on bacteremia due to ventilation-induced translocation in an ARDS rat model with *Klebsiella*-induced pneumonia. After whole lung lavage Sprague-Dawley rats intravenously received either a high or a low dose of an immunoglobulin preparation, or an albumin solution as control, followed by an intratracheal injection of a *Klebsiella pneumoniae* solution. Blood Colony Forming Units (CFUs) in the treatment groups were significantly lower during the 3-h ventilation period compared to the control group. We conclude that IgM-enriched polyclonal immunoglobulins lead to a reduction of bacteria in blood of surfactant-deficient, ventilated rats infected with *Klebsiella pneumoniae*.

Introduction

Ventilated patients suffering from acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) often develop pneumonia ¹ and may eventually die because of sepsis and multi organ failure (MOF) ².

It has been demonstrated that mechanical ventilation with high tidal volumes or high peak inspiratory pressures (PIP) and little or no positive end-expiratory pressure (PEEP) may induce decompartmentalisation of bacteria ³⁻⁵ and cytokines ⁶ from the lung into the systemic circulation and it becomes increasingly realized that this process plays an important role in the pathophysiology of MOF ^{7, 8}.

The ever increasing antimicrobial resistance worldwide is causing a serious threat especially to critically ill patients on intensive care units (ICUs) where nosocomial infections and ventilator-associated pneumonia (VAP) are a common problem ^{9, 10}.

Antibiotics alone may no longer be sufficient to counter this threat. A promising supplement to antibiotic therapy that may be able to deal with this problem are intravenous (i.v.) polyvalent immunoglobulin preparations containing IgA, IgG and IgM. These preparations possess a broad range of opsonizing capacities against most important human pathogens ^{11, 12} and their related exotoxins, superantigens and membrane structures ^{11, 13}. Whether or not immunoglobulins are clinically useful and effective as a therapy for sepsis remains debatable, and convincing beneficial clinical evidence has yet to be found.

Based on the antimicrobial properties of immunoglobulins we investigated the effect of an i.v. administered IgM-enriched polyclonal immunoglobulin preparation on bacteremia due to ventilation-induced bacterial translocation. Therefore we chose an animal model of respiratory failure induced by surfactant depletion and a ventilation setting providing us with the potential to cause bacterial translocation. We hypothesized that a preventive i.v. application of this immunoglobulin preparation would reduce the bacterial blood load in surfactant-deficient ventilated rats infected with *Klebsiella pneumoniae*.

Materials and methods

Animal preparations

The institutional Animal Investigation Committee Care approved the study protocol and handling of the animals was in accordance with the latest European Community guidelines (86/609/EC).

A total of 18 male Sprague-Dawley rats (body weight 270-350 g) were used. Anesthesia was induced with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane, Pharmachemie bv, Haarlem, the Netherlands), a sterile polyethylene catheter was inserted into a carotid artery for arterial blood sampling and a sterile metal cannula was inserted into the trachea. Anesthesia was continued with pentobarbital sodium 60 mg/kg/h bodyweight i.p. (Nembutal[®], Algin bv., Maassluis, the Netherlands). Subsequently, muscle relaxation was induced and sustained with pancuronium bromide 2 mg/kg/h bodyweight i.m. (Pavulon[®], Organon Technika, Boxtel, the Netherlands) followed by connection to the ventilator. Animals were ventilated in parallel in a pressure-controlled time-cycled mode (Siemens Servo 300, Siemens Elema, Solna, Sweden). Initial PIP was 12 cm H₂O with a PEEP of 2 cm H₂O, the fractional inspired oxygen concentration (FiO₂) was set at 1.0, I/E ratio of 1:2; frequency = 30 breaths per min. To re-aerate atelectatic lung areas induced by the surgical procedure, the airway pressure was increased to a PIP of 25 cm H₂O for 3 breaths. The body temperature was kept at 37°C by means of a heating pad.

Lung lavage procedure

All rats were surfactant depleted by whole lung lavage (33 ml warm saline/kg bodyweight) according to Lachmann et al. ¹⁴ until PaO₂ was < 80 mmHg. During the lavage the ventilation pressures were set at a PIP of 26 cm H₂O and a PEEP of 6 cm H₂O. After the lavage procedure ventilator settings were set at a PIP of 30 cm H₂O and a PEEP of 10 cm H₂O and maintained for the remainder of the experiment. Ventilatory rate was adjusted to maintain normocapnia (35-45 mm Hg).

Application of immunoglobulin preparation/albumin

After lavage a sterile i.v. cannula (Neoflon™, Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was inserted into the penile vein of all rats. The animals were then randomly assigned to one of the three groups (n=6 each). In this study we used IgM-enriched polyclonal immunoglobulins (Pentaglobin®[®], Biotest, Dreieich, Germany; antibody titer for *K. pneumoniae* 1:1280/2560 tested with passive haemagglutination). The first group (high dose) received 600 mg/kg immunoglobulins i.v. (50 mg/ml). The second group (low dose) received 300 mg/kg immunoglobulins (50 mg/ml) and 115 mg/kg bovine albumin solution (19 mg/ml). The third group received 230 mg/kg bovine albumin solution (19 mg/ml) only and served as control group. The colloid osmotic pressure of the albumin solution was adjusted to be equivalent to the 50 mg/ml immunoglobulin preparation. Solutions were slowly injected through the i.v. cannula over a 15-min period.

Induction of pneumonia

The inoculum of *K. pneumoniae* was aerosolised intratracheally using a miniature nebulizer (Penn-Century, Philadelphia, PA, USA). Animals were subsequently ventilated for 3 h.

The inoculum (1 µl/g rat weight of saline with a concentration of 10⁸ colony forming units (CFU)/ml *K. pneumoniae*) was prepared as follows: stationary-phase cultures were prepared by incubation for 16 h at 37°C in Mueller-Hinton broth (MHB) with a standard *K. pneumoniae* solution³. Thereafter, 200 µl of this broth was taken and combined with 19.8 ml MHB and incubated for 2.5 h at 37°C. One ml of this suspension was centrifuged at 1300 g for 2 min, the supernatant removed and the pellet resuspended in 1 ml saline yielding the final concentration of 10⁸ CFU/ml. The inoculum was stored on ice until use.

To verify the number of viable bacteria in the inoculum, 100 µl of 10-fold dilution steps in saline were plated on blood agar plates and incubated overnight at 37°C. CFUs were visually counted the following day.

Measurements

Arterial blood gases (PaO₂, PaCO₂) were measured with conventional methods (ABL 555, Radiometer Copenhagen, Denmark) just before the lung lavage (before lavage), immediately after lavage (after lavage, t₀) and at 1 (t₁), 2 (t₂) and 3 (t₃) h after lavage and mechanical ventilation. To replace the blood loss caused by blood sampling, all animals received 5 ml/kg Hemohees 6% (Plasma expander containing hydroxyethyl starch, molecular weight 200000, Braun Melsungen, Melsungen, Germany) intra-arterially after sampling at t₀, t₁ and t₂. Mean

arterial pressure (MAP) was measured in one of the carotid arteries before lavage, after lavage and at 15 min, 1, 2 and 3 h using a transducer (Sirecust 1280, Siemens, Danvers, MA, USA).

Blood samples of 1 ml were taken before lavage and at 1, 2 and 3 h after lavage, and cultured undiluted onto blood agar plates (Becton Dickinson, Alphen a/d Rijn, the Netherlands) and incubated at 37°C. *K. pneumoniae* CFUs were counted after 24 h, the lower limit of detection by this method was 1 CFU/ml.

After 3 h of ventilation all animals were killed with an overdose of pentobarbital sodium. Lungs were taken sterile from the thorax, followed by a broncho-alveolar lavage (BAL) (30 ml/kg). Then lungs were weighed and homogenized in 20 ml sterile saline for 2 min at 40,000 rpm in a tissue homogenizer (Virtis “23”, The Virtis Company, NY, USA). The number of viable bacteria in the lung homogenates and BAL was determined by plating 10-fold dilution steps on blood agar plates. These plates were incubated at 37°C, *K. pneumoniae* CFUs were counted after 24 h.

Statistic analysis

Data are reported as mean \pm standard deviation (SD). Log transformed CFU counts (instilled CFUs, lung and BAL), generic data, blood CFUs, PaO₂, PaCO₂ and MAP were compared with one-way and repeated measures ANOVA with a Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$.

Results

At the start of the experiment the bodyweight of the 18 rats was comparable in all three groups. All animals survived the 3-h study period.

Verification of the number of viable bacteria in the inoculum showed that all rats received between 1.8×10^8 and 3.7×10^8 CFUs per ml *Klebsiella* solution. Each rat was inoculated with 1 μ l of this solution per g rat weight. There was no significant difference between the three groups in the amount of *K. pneumoniae* the rats received.

At t0 all rats tested negative for *K. pneumoniae* in blood. Figure 1 shows blood CFUs of the three groups after 1, 2 and 3 h of ventilation. The control group had significantly ($p < 0.05$) higher blood CFUs after 1 and 2 h compared with the low dose and high dose groups. After 3 h the control group had significantly ($p < 0.05$) higher blood CFUs compared

with the high dose group. In all 3 groups there was a significant increase in blood CFUs during the 3-h study period.

The number of total lung *K. pneumoniae* CFUs calculated from lung homogenates and BAL at 3 h was similar in all groups. Table 1 presents data on PaO₂, PaCO₂ and MAP. Table 2 gives generic and CFU data; significant differences are indicated.

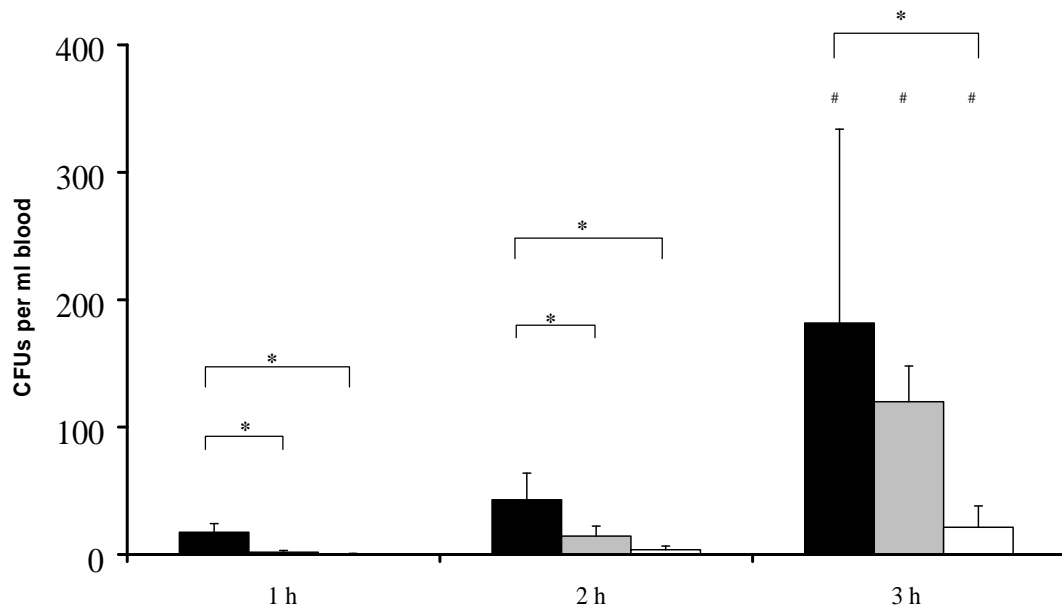


Figure 1. CFUs in arterial blood after 1, 2 and 3 h ventilation. Data are mean \pm SD from groups of 6 animals: control (black bar), low dose (gray bar) and high dose (open bar). Significant difference ($p < 0.05$) between groups: control vs. low dose and/or high dose (*). Significant difference ($p < 0.05$) within all groups: t=1 h and t=2 h vs. t=3 h (#).

Table 1. Data on blood gas tension and mean arterial pressure

		Control	Low dose	High dose
PaO ₂	before lavage	557 ± 37.8	556 ± 23.7	553 ± 19.0
	after lavage	64 ± 7.3 ^a	65 ± 8.6 ^a	71 ± 10.6 ^a
	1 h	150 ± 65.7 ^{a,b}	88 ± 29.3 ^a	125 ± 76.0 ^a
	2 h	90 ± 27.1 ^a	73 ± 17.1 ^a	86 ± 34.6 ^a
	3 h	76 ± 13.6 ^{a,d}	81 ± 44.8 ^a	56 ± 9.5 ^a
PaCO ₂	before lavage	38 ± 6.2	36 ± 1.5	35 ± 6.1
	after lavage	52 ± 8.6	54 ± 11.4 ^a	48 ± 7.7
	1 h	45 ± 10.3	58 ± 5.4 ^a	51 ± 8.9 ^a
	2 h	48 ± 6.8	57 ± 10.4 ^a	47 ± 7.7
	3 h	44 ± 9.0	51 ± 11.8	54 ± 9.3 ^a
MAP	before lavage	154 ± 31.3	142 ± 29.1	157 ± 14.3
	after lavage	123 ± 26.8	138 ± 15.9	147 ± 13.3
	15 min	145 ± 30.4	140 ± 28.5	154 ± 19.0
	1 h	135 ± 16.4	119 ± 32.8	144 ± 20.0
	2 h	91 ± 27.9 ^{a,c}	104 ± 34.8	96 ± 24.0 ^{a,b,c,d}
3 h	86 ± 35.3 ^{a,c,d}	74 ± 33.9 ^{a,b,c}	87 ± 34.2 ^{a,b,c,d}	

Data reported as mean ± SD, PaO₂ and PaCO₂ (torr), mean arterial pressure (MAP) (mmHg). There were no significant differences between groups. Significant difference within groups (p < 0.05): before lavage vs. other (^a); after lavage vs. other (^b); 15 min vs. other (^c); 1 h vs. other (^d).

Table 2. Generic data and colony forming units

	Control	Low dose	High dose
Bodyweight (g)	336.7 ± 19.3	329.8 ± 11.8	308.4 ± 23.2
Lung weight (g)	4.4 ± 0.9	3.6 ± 0.3	3.8 ± 0.3
Instilled CFUs	7.93 ± 0.10	7.98 ± 0.09	7.93 ± 0.15
Total Lung CFUs	8.7 ± 0.3	8.8 ± 0.2	8.4 ± 0.6

CFUs are log transformed, measured in instilled CFU solution (Instilled CFUs) and in total lung CFUs and in the whole lung (Total Lung CFUs) of the 3 groups (mean ± SD). Significant difference ($p < 0.05$) low dose vs. high dose (*)

Discussion

The results show that an i.v. application of immunoglobulins containing IgA, IgG and IgM significantly reduces bacteremia caused by ventilation-induced bacterial translocation in surfactant-deficient, ventilated rats.

Bacterial translocation from the lung into the systemic circulation caused by ventilation involving high lung volumes and repetitive alveolar collapse was first demonstrated by Nahum et al. ⁴ and Verbrugge et al. ³. In contrast to their studies in healthy animals, we chose a model of ALI/ARDS induced by repeated lung lavages ¹⁴. In this ALI/ARDS model, extensive damage to the alveolar-capillary membrane is induced by shear forces due to repeated alveolar closure and reopening of alveoli ^{15, 16}. Such damage is characterized by the loss of alveolar barrier function caused by disruption of alveolar epithelium and endothelium. These alterations to the alveolar-capillary membrane are thought to be the main route for bacteria in the lung to gain access to the systemic circulation ^{17, 18}. In the current study, after inducing ALI/ARDS all animals were infected with *K. pneumoniae* and ventilated with the same pressures (PIP of 30 cm H₂O; PEEP of 10 cm H₂O). These seemingly high PIP and PEEP values in this model were required for the rats to survive the 3-h study period in terms of adequate blood oxygenation and CO₂ elimination. Because PaO₂ values were comparable between the three groups throughout the study, the immunoglobulin preparation appeared to have no influence on lung function as characterized by gas exchange.

To find a treatment other than antibiotics for ventilation-induced bacteremia, we administered immunoglobulins i.v. in two different dosages before infection with *K. pneumoniae*. In vitro studies have shown that IgM-enriched immunoglobulins are able to bind and neutralize a broad range of human relevant pathogens and their related super antigens, membrane structures and exotoxins [11-13](#). By blocking complement activation without interfering with the clearance of bacteria, immunoglobulins were also shown to have direct anti-inflammatory properties [19](#). The half-life of the immunoglobulin preparation we used varies according to the different isotypes and ranges from about three weeks (IgG and IgA) to 3-5 days (IgM). Because the present study lasted only 3 h half-life should not have influenced our results.

Whether or not immunoglobulins are useful and effective as a therapy for sepsis remains debatable. Since 1985, several placebo-controlled clinical studies investigated that issue with controversial results. Most of them had only a small patient population. Nevertheless, some investigators were able to show possible beneficial effects of i.v. immunoglobulins on patient survival [20-22](#). However, no convincing clinical evidence has yet been found for the usefulness of i.v. immunoglobulins in sepsis, a finding that was recently confirmed by Tugrul and colleagues [23](#). A reason for this controversy could lie in the different application timings in relation to the disease stage. For example, Tugrul and colleagues started the intervention when severe sepsis had already developed so that the patient was already highly immune-compromised. At this stage phagocytosis and intracellular killing are severely impaired so that the host is no longer able to remove the immune complexes that form upon binding of the specific antibodies to their targets [24](#). Therefore, for future research, intervention timing should be more standardized in the way of giving immunoglobulins patients at risk as early as possible in order to achieve beneficial effects.

The present study showed a significant reduction in CFUs in the blood of animals treated with our immunoglobulin preparation. Moreover, this reduction was dependent on the immunoglobulin dose received. After 3 h of ventilation this dose-dependency reached significant levels.

We also determined the number of CFUs in the whole lung to study the effect of i.v. immunoglobulins on bacterial growth in the infected organ. Because there were no significant differences in the total lung CFU counts between the 3 groups, the immunoglobulin preparation appeared to have no influence on the growth of *K. pneumoniae* in the lung. All animals had a significant trend of a decrease in blood pressure after 2 to 3 h of ventilation and

infection with *K. pneumoniae*, indicating the onset of septic vasodilation. Even though our immunoglobulin preparation neutralized a great amount of translocated bacteria it failed to prevent a drop in blood pressure during the 3-h study period.

The lung is a potential source of bacteremia and endotoxemia in intubated patients suffering from pneumonia. The prevalence of ventilator-associated pneumonia ranges from 9 to 70% depending on the population studied, the type of ICU, and the diagnostic criteria used [10](#). The increasing number of multi-drug-resistant human pathogens are a great threat, especially to severely ill/immune-compromised patients. Unlike antibiotics, bacteria have no direct means of developing resistance against antibodies. This study indicates that immunoglobulins may prove useful to reduce the bacterial blood load in patients with ventilation-induced lung injury, ALI or ARDS. However, extrapolating this very short experiment into a clinical setting may be difficult for a number of reasons. Animal experiments can never replace clinical trials, but may serve as an indicator of whether it may be worthwhile to conduct clinical trials. Animal models allow us to create well-defined experimental conditions in contrast to the complex clinical environment where many parameters may influence the total clinical picture. In order to obtain clear results we used an extreme model, with the animals being severely sick even before they received a massive dose of bacteria, a setting that probably will never occur in a clinical environment. Also we used a completely preventive approach giving the immunoglobulins before infecting the animals in contrast to the clinical procedure where immunoglobulins are mostly used as a treatment rather than as a prophylaxis of sepsis.

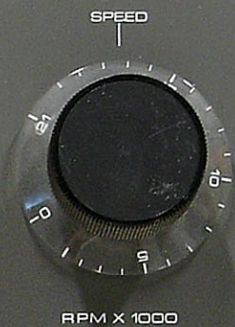
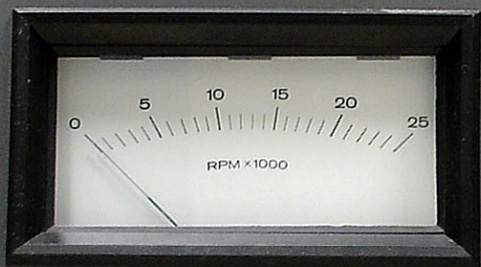
Conclusion

Our study in rats shows that i.v. administered IgM-enriched immunoglobulins can largely neutralize *K. pneumoniae* translocated from the lung into the bloodstream in a dose-dependent way. However, our study failed to show other direct protective effects on PaO₂, PaCO₂ and MAP, or on bacterial growth in the lung within the 3-h study period of these experiments.

In view of the high incidence and prevalence of ventilator-associated pneumonia, our finding that IgM-enriched immunoglobulins can reduce blood CFUs is relevant for the clinical setting because ventilated ARDS patients often develop both pneumonia and sepsis. Lowering the bacterial blood load by means of i.v. immunoglobulin treatment might therefore prevent further secondary injuries in septic patients.

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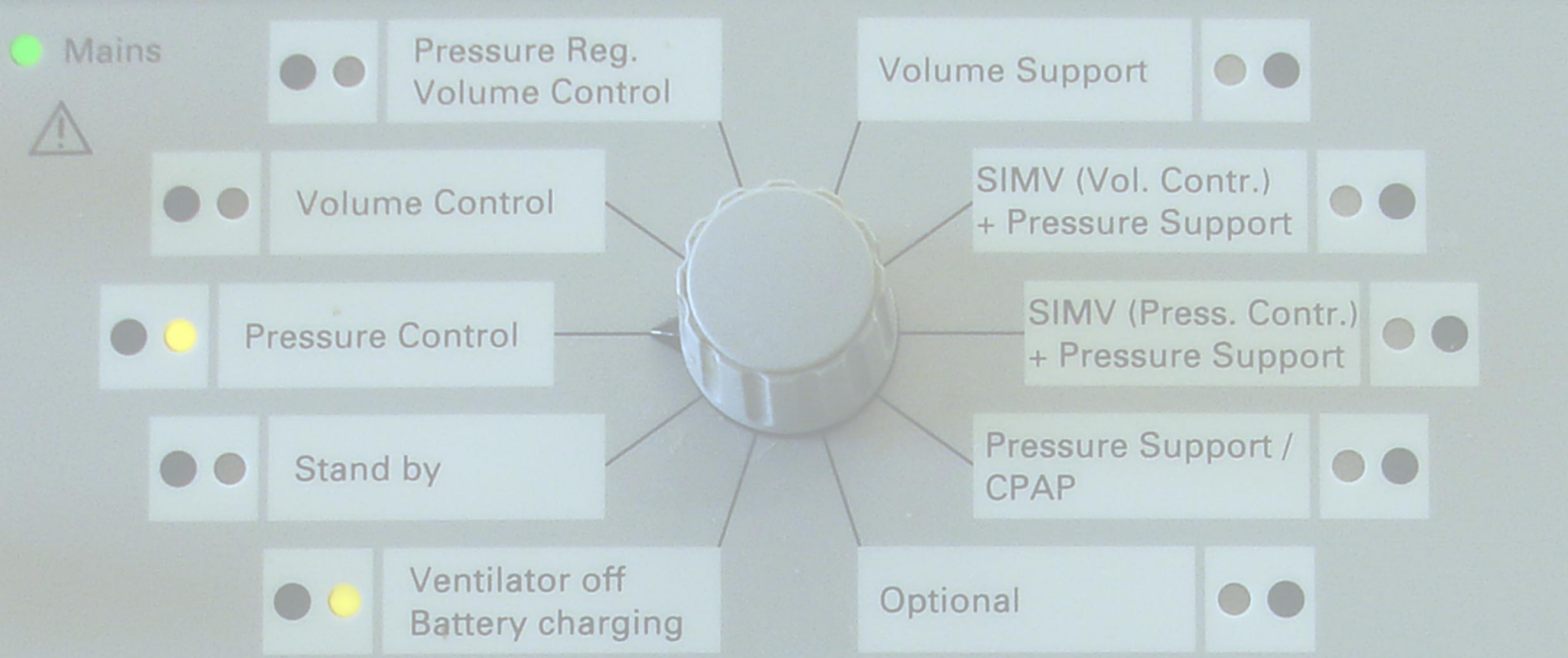
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Chapter

8

Local versus systemic antimicrobial (tobramycin) treatment in a pneumonia rat model

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Abstract

This study compared the efficacy of intratracheal tobramycin with and without surfactant as carrier to intravenous tobramycin treatment in rats with a respiratory *Klebsiella pneumoniae* infection. Therefore, rats received six hours after infection with *Klebsiella pneumoniae* intratracheal (with or without surfactant) or intravenous antibiotic treatment. At 24 and 48 hours post infection, surviving rats were sacrificed and lung and blood colony forming units were determined. Only rats treated with surfactant-tobramycin were able to clear bacteria from their lungs. We conclude that the used dose of intratracheal tobramycin with surfactant is superior in clearing bacteria from lungs compared to the same dose of tobramycin given intravenously.

Introduction

Pneumonia is still an important cause of morbidity and mortality on intensive care units where nosocomial infections and ventilator-associated pneumonia are a common problem [1, 2](#). Antibiotic treatment of these lower respiratory tract infections is complicated by the ever-increasing antimicrobial resistance worldwide leading to the necessity of using more human-toxic antibacterial agents and therefore increasing the risk of adverse side effects, such as oto and nephrotoxicity caused by aminoglycosides [3](#). Application of antibiotics directly to the region of infection might reduce these systemic adverse effects. So far, aerosols [4-9](#) and direct endotracheal instillations [4-6, 10](#) have been used for intratracheal antibiotic application with varying success. While earlier studies showed a low efficacy of aerosols that was probably caused by the limited deposition of antibiotics in the lung, especially in the non-ventilated areas [5, 9, 11, 12](#), more recent studies using ultrasonic nebulizers could demonstrate better efficacy of aerolized antibiotics compared to intravenous treatment [13, 14](#). Antibiotics administered directly into the trachea will most likely not reach the small peripheral airways, due to the high surface tension of the instilled fluids (e.g. water, saline) [4, 15, 16](#). Because surfactant has the ability to lower surface tension and has excellent spreading properties [17](#), it has been suggested to use exogenous surfactant as a drug delivery system for antibiotics to the alveolar lung compartment [16, 18, 19](#). Van 't Veen and colleagues demonstrated that intratracheal tobramycin mixed with surfactant significantly improved survival of mice with pulmonary *Klebsiella* infection compared to intratracheal tobramycin alone [20](#). However, to our knowledge, nothing is known about the efficacy of intratracheal surfactant-antibiotic application as compared to intravenous antibiotic treatment. Even though the advantages of local administration are obvious, it must be proven that it is at least as effective as today's standard systemic therapy to become a reasonable alternative. Therefore, the aim of the present study was to test whether local antibiotic application using surfactant as carrier can be equally or even more effective compared to intravenous treatment in clearing lung bacteria in a *Klebsiella* pneumonia rat model.

Material and Methods

The institutional Animal Investigation Committee Care approved the study protocol and handling of the animals was in accordance with the latest European Community guidelines (86/609/EC).

Induction of pneumonia

Anaesthesia was induced in 90 male Sprague-Dawley rats with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane, Pharmachemie bv, Haarlem, The Netherlands). Anesthetized rats were intubated and the inoculum of *K. pneumoniae* was instilled intratracheally. Each rat received 1 µl inoculum/g body weight containing $\sim 10^8$ colony forming units (CFUs) per ml saline. The inoculum with the *K. pneumoniae* suspension was prepared as follows: stationary-phase cultures were prepared by incubation for 16 h at 37°C in Mueller-Hinton broth (MHB) with a standard *K. pneumoniae* solution. Thereafter, 200 µl of this broth was taken and combined with 19.8 ml MHB and incubated for 2.5 h at 37°C. One ml of this suspension was centrifuged at 1300 g for 2 min, the supernatant removed and the pellet resuspended in 1 ml saline yielding the final concentration of 10^8 CFU/ml. The inoculum was stored on ice until use. To verify the number of viable bacteria in the inoculum, 100 µl of 10-fold dilution steps in saline were plated on blood agar plates and incubated overnight at 37°C. CFUs were visually counted the following day.

Study groups

Six hours after infection with *K. pneumoniae*, the rats were randomized to 7 different groups as shown in Table 1. Following anaesthesia and treatment, the animals (including control animals) of each group were randomly assigned to subgroups with an observation period of 24 h and/or 48 h. The surfactant (HL-10, HALAS Pharma GmbH, Oldenburg, Germany) was suspended in 0.2 M NaHCO₃ (pH 8.3) based on earlier work done by van 't Veen et al. [20](#). Tobramycin (tobramycin, Centrafarm, Etten-Leur, The Netherlands, 40 mg/ml) was diluted in 0.2 M NaHCO₃ (pH 8.3) [20](#) to obtain a concentration of 4 mg/ml. Surfactant and tobramycin were mixed before administration to the tobramycin-surfactant animals. Intratracheal instillation was performed by means of intubation. The rats of the intravenous tobramycin group received tobramycin as an injection into the penile vein. Animals received 150 mg/kg surfactant and/or 1 mg/kg tobramycin yielding a total treatment volume of 3.7 ml/kg (either intravenous or intratracheal) for each rat. All animals were given intramuscular analgesics (Temgesic®, 0.1 mg/kg, Schering-Plough bv, Utrecht, The Netherlands) with the exception of the *Klebsiella* growth control animals which were killed 6 hours after infection. Their lungs were removed, weighed, homogenized and the homogenate plated on blood agar plates. Unpublished data on rat lung weight was used for healthy baseline values. The animals in the remaining groups were replaced into flow-cap cages and received food and water ad libidum.

Table 1. Study groups

Group	n	Treatment	Application method	Observation period (hours)
Control (C): 24/48	8 / 7	none	none	24 / 48
Surfactant (S): 24/48	8 / 9	surfactant *	intratracheally	24 / 48
Tobramycin (T): 24/48	8 / 8	tobramycin #	intratracheally	24 / 48
Tobramycin i.v. (Tiv): 24/48	7 / 8	tobramycin #	intravenously	24 / 48
Tobramycin +Surfactant (TS): 24/48	8 / 8	tobramycin + surfactant *,#	intratracheally	24 / 48
Saline:	5	saline \$	intratracheally	48
Growth control:	6	none	none	6

* surfactant 150 mg/kg; 40 mg/ml, # tobramycin 1 mg/kg, \$ saline 0.2 M NaHCO₃ (pH 8.3)

Blood CFUs

At 24 or 48 hours after bacterial instillation anaesthesia was again induced as described above. A sterile polyethylene catheter was inserted into a carotid artery for arterial blood sampling. Arterial blood (1 ml) was drawn and cultured either undiluted or diluted (10-fold dilution steps) on blood agar plates depending on the expected range of CFUs in blood of each study group. *K. pneumoniae* CFUs were counted visually after 24 h.

Lung CFUs

Lungs were removed from the thorax, weighed and homogenized in 20 ml sterile saline for 2 min at 40000 rpm using a tissue homogenizer (Virtis “23”, The Virtis Company, NY, USA). The number of viable bacteria in the lung homogenates was determined by plating 10-fold dilution steps on blood agar plates. These plates were incubated at 37°C, *K. pneumoniae* CFUs were counted visually after 24 h.

Statistical analysis

Data on survival are expressed as percentage of all animals per group; data were analyzed using Fisher’s exact test. All other data are reported as mean ± standard deviation (SD). Intergroup differences were compared with an unpaired t-test or ANOVA followed by the Turkey post hoc test. A Kruskal-Wallis test with Dunn post hoc test was used to analyze blood CFUs. Differences in bacterial growth in rat lungs were analyzed with a paired t-test.

Apart from survival, the S 48 group was not included in the statistical analysis because only 3 rats survived the second day. Statistical significance was accepted at p-values < 0.05.

Results

Baseline characteristics

The mean weight of the studied animals was 303 ± 24 g and there were no significant intergroup differences. All rats were inoculated intratracheally with 7.7 ± 0.1 CFUs (\log_{10}). The mean volume of treatment solutions (either intravenous or intratracheal) was 1.1 ± 0.1 ml. There was no significant difference in the volume and amount of bacteria each rat received (Figure 1).

Survival

All but 1 animal in the control group survived the 48-hour study period after pneumonia induction (Table 2). As expected, this was also the case for animals treated with tobramycin, administered either intravenously or endotracheally (with or without surfactant). It was interesting to see that significantly more animals died in the 48-hour observation period if they had not received tobramycin but either surfactant or saline, the latter having a mortality rate of 100%.

Lung CFUs

The CFU counts of growth control animals revealed a significant increase of *K. pneumoniae* during the first 6 hour post infection, pre-treatment period (instilled 7.7 ± 0.1 ; recovered: $8 \pm 0.1 \log_{10}$ CFUs, $p < 0.05$, $n=6$). Surfactant and control animals showed an increase in lung CFUs after both 24 and 48 hours. Intravenous tobramycin treatment attenuated this growth but could not entirely prevent it, as shown by the significant increase of bacteria after 24 hours compared to instilled CFUs.

As can be seen in Figure 1, only surfactant-tobramycin treatment resulted in clearance of CFUs from infected lungs. Lung and blood CFUs of saline-treated animals could not be determined because all rats of this group died unobserved during the night.

The total lung weight corrected for body weight was significantly higher in the surfactant and control groups (including the growth control group, 7.7 ± 0.6 g) after 24 hours compared to healthy controls (5.8 ± 0.6 g). At the end of the 48 hour observation period, all groups had significantly heavier lungs compared to the healthy controls (Table 2).

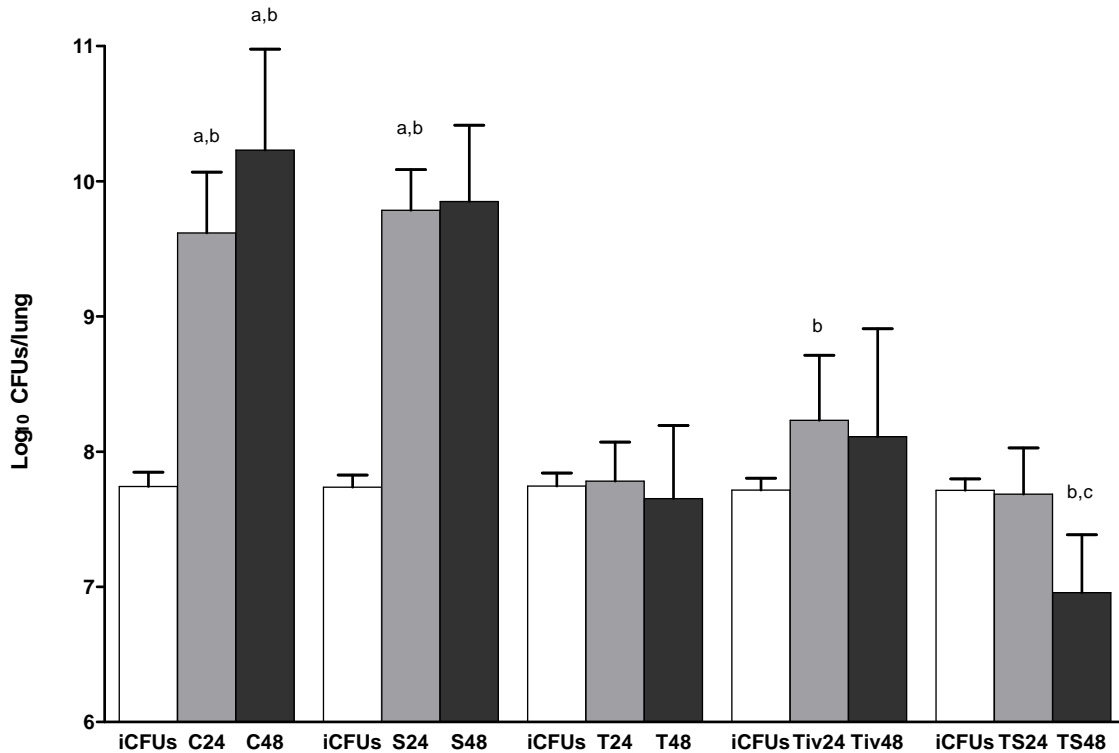


Figure 1. CFUs in lung homogenate (mean \pm SD, \log_{10}). The open bars show the number of instilled CFUs (iCFUs) for each group pair. ^a $p < 0.001$ vs. T24 and 48, Tiv24 and 48, TS24 and 48; ^b $p < 0.05$ vs. iCFUs; ^c $p < 0.01$ vs. Tiv24 and 48. For definitions of group classifications see Table 1.

Blood CFUs

There was no significant difference between the groups in the number of *K. pneumonia* blood positive animals (Table 2). As shown in Figure 2, after one and two days the number of CFUs in blood cultures differed greatly for each animal per group, with control and surfactant groups having the highest counts. Compared to these two groups tobramycin treatment (either intravenous or intratracheal) attenuated the degree of bacteremia but this was only significant when compared to the tobramycin-surfactant group.

Table 2. Data on survival, blood CFUs and lung weight.

	C24 / 48	S24 / 48	T24 / 48	Tiv24 / 48	TS24 / 48
Survival, %	100 ^a / 86 ^a	88 ^a / 33 ^b	100 ^a / 100 ^a	88 ^a / 100 ^a	100 ^a / 100 ^a
Blood pos, %	88 / 100	86 / 100	57 / 100	57 / 63	63 / 88
WL, g/kg	7.8 \pm 0.7* / 13 \pm 5.0*	8.2 \pm 0.7* / 11 \pm 5.0	7.1 \pm 0.6 / 8.9 \pm 1.3*	6.7 \pm 0.5 / 8.9 \pm 1.0*	7.2 \pm 0.7 / 8.7 \pm 0.9*

Definitions of abbreviations: WL = weight of lung corrected for body weight (mean \pm SD); Blood pos = % blood CFU positive animals; Survival = % animals alive. For definitions of group classifications see Table 1. * $p < 0.05$ vs healthy control animals, ^a $p < 0.05$ vs. Saline (Saline = 100% mortality), ^b $p < 0.05$ vs. TS48, Tiv48, T48

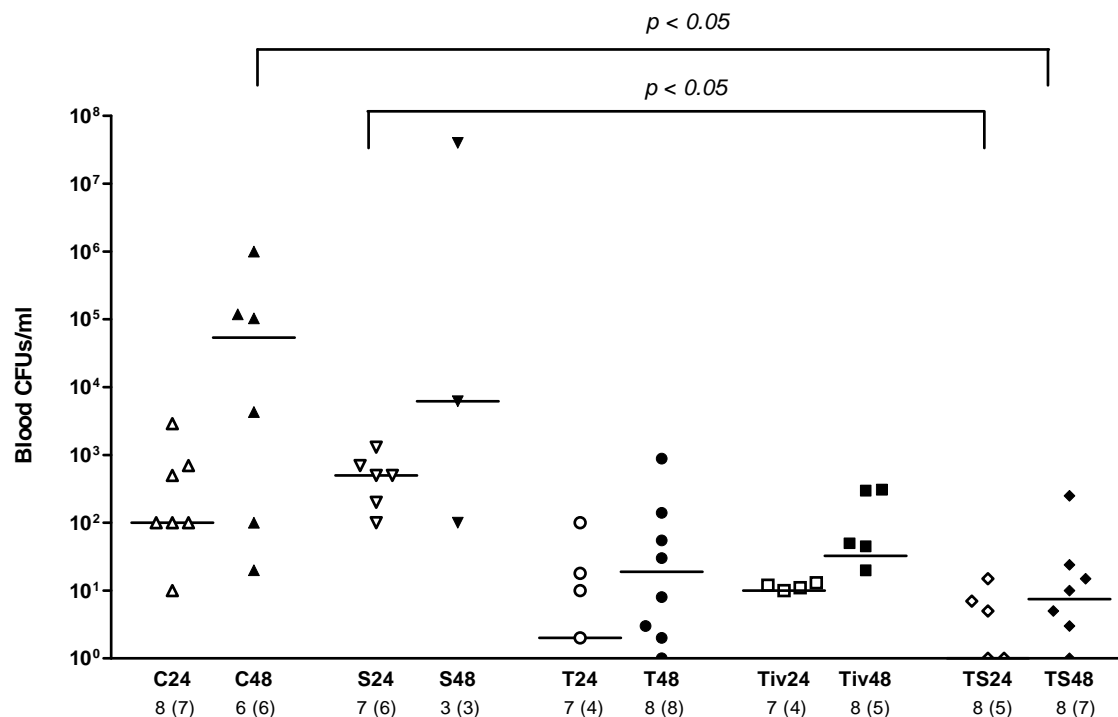


Figure 2. Scatter plot of individual CFUs in arterial blood at the end of the experiment. The median is presented as a solid line (including animals with negative cultures). The numbers below the names of the groups indicate the number of surviving animals and (in brackets) the number of animals with a positive blood culture. Animals with at least 1 CFU are shown. Significant differences are indicated. For definitions of group classifications see Table 1.

Discussion

The results of this study show that a single intratracheally administered dose of the antibiotic tobramycin mixed with exogenous surfactant is able to clear bacteria in rat lungs infected with *K. pneumoniae*. On the other hand, intravenous treatment using the same tobramycin dose led to a growth of *K. pneumoniae* in lungs of infected animals. This suggests that a given antimicrobial dose is more effective in clearing lung bacteria when applied intratracheally using surfactant as a carrier as compared to systemic application.

A previous study showed that surfactant used as a carrier for intratracheal tobramycin reduced mortality compared to no treatment, intratracheal surfactant or tobramycin alone in a mouse model of *Klebsiella pneumoniae* [20](#). It was suggested that the improved survival is due to the superior spreading properties of surfactant which, when used as a carrier, enables the intracheal tobramycin to be more widely and peripherally distributed throughout the infected lung [15, 17, 20-22](#). The present study confirms this suggestion by showing for the first time that surfactant-tobramycin in fact reverses bacterial growth in affected lungs. But more

importantly, the present study also shows that intratracheal application in combination with surfactant may be a reasonable alternative to intravenous therapy by proving its efficacy even at a non-clinical lower tobramycin dose where intravenous treatment failed to prevent bacterial growth. A possible explanation for this failure could be that the intravenous dose of tobramycin used might have been too small to reach effective concentrations at the site of infection. Studies have shown that intravenously administered tobramycin accumulates only in the kidneys and bladder (within 15 min) with only 0.8% of the original concentration present in the lung after 6 hours ²². The tobramycin dose (1 mg/kg) used in the present study was derived from pilot studies and represents the minimal effective dose for intratracheally administered surfactant-tobramycin which is lower than the usual clinical dose of about 10 mg/kg. It is plausible that increasing the intravenous dose might have increased efficacy, but most likely only at the cost of an increased risk of unwanted adverse side effects. Interestingly, the major differences in lung CFUs did not translate into different occurrences of bacteremia between the groups. However, compared to the control and surfactant groups, there was a trend towards lower numbers of bacteria in blood when tobramycin was used for treatment either intravenously or intratracheally. The fact that only surfactant-tobramycin therapy could produce significant decreases in blood CFU counts seems to further suggest the better efficacy of this application method. Despite the differences in bacterial growth in lungs, we found comparable mortality rates in the animals treated with tobramycin either intravenously or intratracheally with or without surfactant. The latter result is in contrast to the study by Van 't Veen and colleagues ²⁰, who demonstrated decreased mortality rates when combining intratracheal tobramycin with surfactant compared to tobramycin alone; however, their study also showed no differences in survival on day 2 suggesting that the discrepancy in mortality rate between the two studies is most likely due to our shorter study period (i.e. 2 days compared with 8 days in the earlier study). It was interesting to see that surfactant treatment without tobramycin led to a significant increase in lung CFUs and mortality. The latter result was also observed by Van 't Veen ²⁰ who speculated that this might be due to the small surfactant dose they used. The present study used a high dose of surfactant (150 mg/kg) thus ruling out their explanation and also questions the rationale to treat pneumonia with exogenous surfactant proposed by Lachmann et al. ¹⁸. We speculate that the increased mortality results from the extra fluid load derived from the intratracheal treatment itself. In our experiment, all infected animals (24 and 48 hours) including *Klebsiella* growth controls that were killed after 6 hour post *Klebsiella* infection had significantly heavier lungs than healthy controls, indicating the presence of pneumonia-associated edema fluid ²³. Instilling

additional volume into these already fluid loaded lungs might therefore worsen lung function with subsequent higher mortality rates. To test this hypothesis we included an additional group of rats that were instilled with saline alone (0.2 M NaHCO₃) intratracheally 6 hours after infection; none of these animals survived the 48 hours. Since all rats receiving intratracheal treatment were instilled a volume of about 1 ml, we concluded that giving extra fluid (not having a direct antibacterial property) to rats with a *K. pneumonia* infection has detrimental effects on survival. Explanations for this finding and why there was no benefit from surfactant therapy remain speculative. The fact that the animals in the present study were healthy and had an intact surfactant system at the start of the experiments might be important, because it has been shown that surfactant treatment in the surfactant-deficient lung improves bacterial clearance and survival [24](#). The present study has the limitation that it cannot answer questions about the final outcome of tobramycin treatment (e.g. possible complete resolution of pneumonia) due to its short time period. On the other hand, we think it is ethically unsound (having humane endpoints in mind) to prolong these experiments for longer than 48 hours, especially for the control, intravenous and surviving surfactant rats who were all seriously ill.

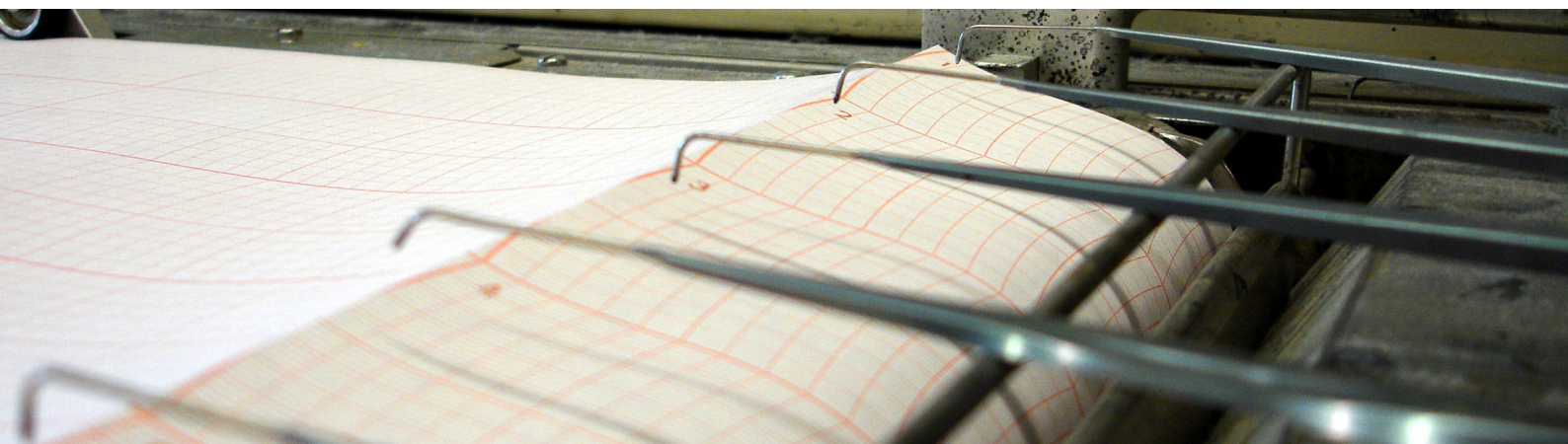
Conclusions

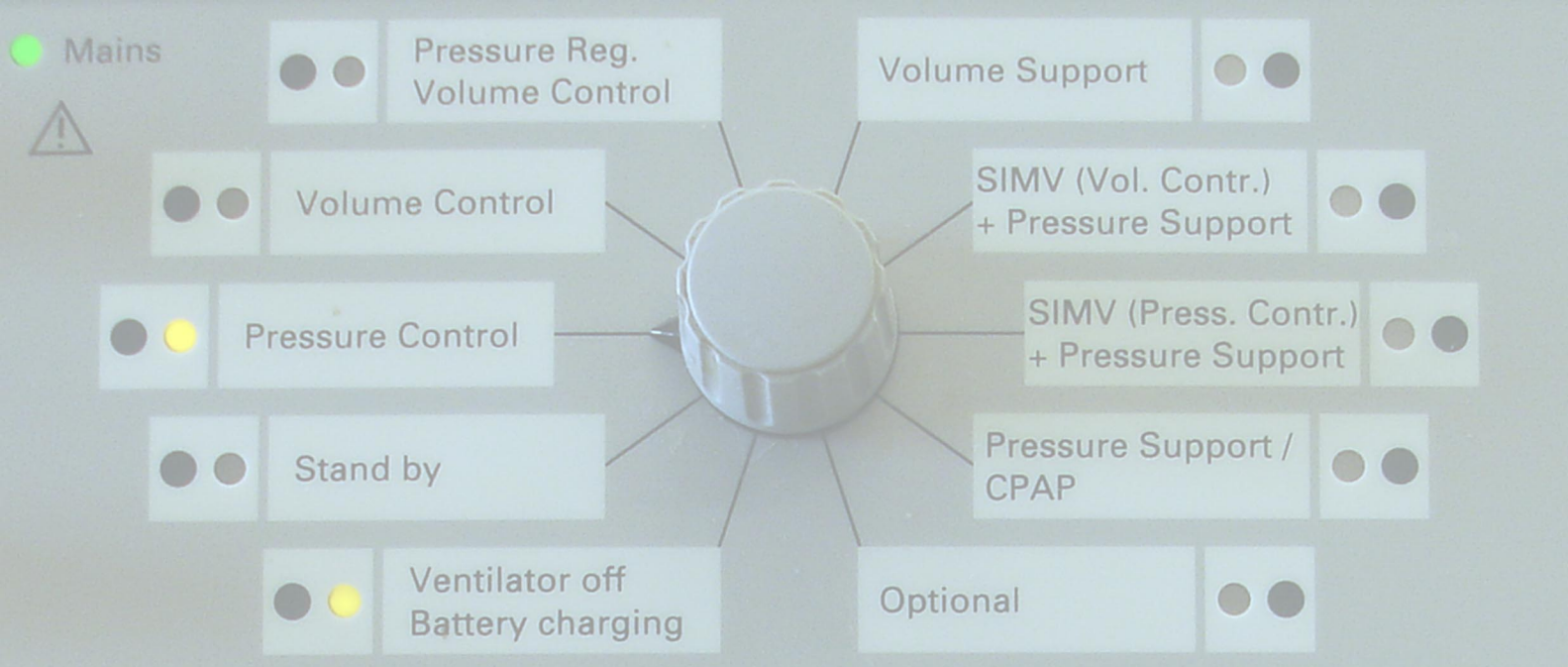
Even though extrapolating data from animal experiments to a clinical setting should always be done with caution, we feel that this study may provide important new insights concerning alternative treatment approaches of pneumonia. Our study suggests that effective intratracheal antibiotic treatment in the lung is feasible using exogenous surfactant. Furthermore, local antimicrobial lung treatment with surfactant was significantly more efficient at the given dose than systemic therapy. Therefore, a higher intravenous dose would be needed to achieve comparable results, increasing the likelihood for systemic side effects even more. We think this could be of clinical relevance for current intensive care units where pneumonia, sepsis and renal failure are closely linked with each other [25-27](#) making the use of systemic antibiotics such as aminoglycosides (with their possible renal toxicity) not a preferable option to treat pneumonia. The failure of exogenous surfactant treatment to show any beneficial effect might influence the discussion on possible future surfactant treatment suggesting a more limited range of surfactant therapy depending on the underlying disease. More studies need to be done to further elucidate this unexpected finding and its precise mechanisms.

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Resp. pattern

Measured freq.
b/min

50

Set freq.
b/min

50

Insp. period
s

0.40

Insp. flow
l/s

Chapter 9

Partial liquid ventilation improves lung function in ventilation-induced lung injury

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Abstract

Background: Disturbances in lung function and lung mechanics are present after ventilation with high peak inspiratory pressures and low levels of PEEP. The combination of perfluorocarbon (PFC) with gas ventilation, better known as partial liquid ventilation (PLV), might be useful for treatment of ventilation-induced lung injury (VILI). Therefore, we investigated whether PLV can re-establish lung function after VILI has been induced.

Methods: Adult rats were exposed to high peak inspiratory pressures without PEEP for 20 min. Thereafter, the animals were randomly divided into five groups. The first group was killed immediately after randomisation and used as a non-ventilated control. The second group received only mechanical ventilation, and three groups received PFC (10 ml/kg, 20 ml/kg, and 20 ml/kg plus 5 ml/kg after one hour to compensate loss of PFC due to evaporation). Then the four groups were mechanically ventilated for two hours. Blood gases, lung mechanics, total protein concentration, minimal surface tension, and small surfactant aggregates/large surfactant aggregates ratio were determined.

Results: PLV improved gas exchange, dose and time dependent, and total lung compliance, but did not decrease the protein concentration or the small aggregates/large aggregates ratio in bronchoalveolar fluid after 2 hours mechanical ventilation.

Conclusion: PLV improves gas exchange and pulmonary compliance in VILI when evaporated PFC is replaced, but does not reduce the level of intra-alveolar protein concentration.

Introduction

It is known that modes of mechanical ventilation which allow end-expiratory alveolar collapse and/or end-inspiratory alveolar overstretching result in a decrease of lung compliance and gas exchange ¹, and lead to atelectasis, pulmonary edema, pneumonitis and fibrosis ^{2,3}. Development of intra-alveolar protein-rich edema in healthy rats subjected to intermittent positive pressure ventilation at high inflation pressures, without positive end-expiratory pressure (PEEP), was first demonstrated by Webb and Tierney ⁴ and was later confirmed by Dreyfuss and colleagues who suggested that high inspiratory lung volumes induce endothelial and epithelial overstretching leading to microvascular injury ^{5,6}. Additionally, it is known that large changes in both volume and surface area result in surfactant depletion from the alveoli into the airways as well as to transformation from surface active large aggregates to inactive small aggregates ⁷⁻¹¹. Thus, loss of surfactant function will increase the surface tension at the air-liquid interface of the alveolar walls resulting in alveolar collapse and an increased suction force on the pulmonary interstitium which causes more alveolar edema. The epithelial/endothelial damage results mainly from the shear forces which appear in a non-homogeneous ventilated lung ^{6,10}. It is known that perfluorocarbons (PFCs) have a surfactant-like activity due to their low surface tension (18 mN/m) which in a surfactant-deficient lung decreases the high surface tension at the air liquid interface ¹²⁻¹⁵. Based on this low surface tension, the resulting peak inspiratory pressures during volume controlled ventilation are reduced ¹²⁻¹⁵. Another property of PFCs is their high density which, mainly in the dependent part of the lung, recruit collapsed alveolar units ¹²⁻¹⁶. The combination of PFCs with gas ventilation, better known as partial liquid ventilation (PLV), finally also improves gas exchange in surfactant deficient lungs ^{12,13}. Additionally, because PFCs might not be affected by the presence of plasma proteins in the alveolus, PFC might prove useful as treatment for VILI ^{17,18}. Therefore, the aim of this study was to establish whether PLV can re-establish lung function in ventilation-induced lung injury.

Material and methods

Animal Preparation

This study was approved by the local Animal Committee at the Erasmus University Rotterdam. The study was performed in 30 adult male Sprague-Dawley rats (body weight 280-350 g). After induction of anesthesia with 2% enflurane and 65% nitrous oxide in oxygen, a polyethylene

catheter was inserted into a carotid artery for drawing arterial blood samples and continuous monitoring of arterial blood pressure. Before tracheostomy, the animals received 30 ml/kg pentobarbital sodium, intraperitoneal (i.p.) (Nembutal[®], Algin BV, Maassluis, the Netherlands). After tracheostomy, muscle relaxation was induced by pancuronium bromide 0.6 ml/kg, intramuscular (i.m.) (Pavulon[®], Organon Teknika, Boxtel, the Netherlands) immediately followed by connection to a ventilator and a pressure transducer for continuous monitoring of arterial blood pressure. The animals were mechanically ventilated with a Servo Ventilator 300 (Siemens-Elema, Solna, Sweden) in a pressure constant time-cycled mode, at an inspired oxygen concentration (FiO₂) of 1.0, frequency of 30 breaths per minute (bpm), peak inspiratory pressure (PIP) of 12 cm H₂O, positive end-expiratory pressure (PEEP) of 2 cm H₂O, and inspiratory/expiratory (I/E) ratio of 1:2. Anesthesia was maintained with pentobarbital sodium 30 ml/kg/h, i.p.; muscle relaxation was maintained with pancuronium bromide 0.6 ml/kg/h i.m. Body temperature was kept within normal range by means of a heating pad.

Experimental Design

In order to induce VILI, PIP was increased to 45 cm H₂O and PEEP was decreased to zero for 20 min, whereas the other ventilator settings were not changed. Thereafter, PIP was decreased to 26 cm H₂O and PEEP was increased to 6 cm H₂O for 5 min. Then, the animals were disconnected from the ventilator to ambient pressure to allow some edema fluid (1-2 ml) to flow from the lungs; after this procedure the animals were randomized.

Experimental Groups

The animals were randomised to one of five groups (n=6 per group). In the first group (Non-Ventilated) the animals were killed after the 5 minute ventilation period of 26/6 (PIP/PEEP) with an overdose of pentobarbital and were used as a non-treated, non-ventilated control group. The second group (Ventilated) received a sham bolus of air 28 ml/kg intra-tracheally and was mechanically ventilated at a PIP of 30 cm H₂O, PEEP of 10 cm H₂O, I/E ratio of 1:2, FiO₂ 1.0, and respiratory rate of 40 bpm for two hours. These ventilator settings were chosen based on results of a preliminary study which showed that applied ventilation pressures of 26/6 cm H₂O (PIP, PEEP, respectively) and 28/8 cm H₂O were too low to keep animals alive for an observation period of 2 hours. Three groups received PFC at a dose of : 10 ml/kg (PFC₁₀), 20 ml/kg (PFC₂₀), or 20 ml/kg plus an extra dose of 5 ml/kg (PFC_{20+R}).

Treatment with PFC

The PFC used in this study (Liquivent[®], Alliance Pharmaceutical, San Diego, CA, USA) is insoluble in water, has a specific gravity of 1.918 g/cm at 25°C, a surface tension of 18.1 dynes/cm, vapor pressure of 3.6 kPa at 20°C and 10.5 kPa at 37°C, an oxygen solubility of 53 ml/100 ml and CO₂ solubility of 210 ml/100 ml at 37°C, at 1 atmosphere pressure [12](#), [13](#). The groups PFC₁₀ and PFC₂₀ received a single dose of PFC intratracheally. The PFC_{20+R} group received an initial dose of 20 ml/kg of PFC and, after 60 minutes, an extra dose of 5 ml/kg of PFC was instilled intratracheally to compensate loss of PFC due to evaporation. At instillation animals were disconnected from the ventilator and PFC was administered directly into the endotracheal tube over 3 to 5 sec; the animals were then immediately reconnected to the ventilator.

Gas Exchange and Hemodynamics

Arterial blood gas samples were taken in all groups before, after VILI, and at 5 min after the 26/6 period, and in the four ventilated groups at 5 min after the 30/10 period, and every 30 min for 2 h. The samples were analyzed for arterial oxygen tension (PaO₂) and arterial carbon dioxide tension (PaCO₂) by conventional methods (ABL 505, Radiometer, Copenhagen, Denmark). At the same time points, arterial pressure was recorded. Hemodynamic support was provided by infusion of 1 ml of saline (to a maximum of 2 ml per hour) when mean arterial pressure (MAP) decreased below 60 mmHg.

Pressure-Volume (P-V) Curves

At 120 min after administration of PFC all animals were killed with an overdose of pentobarbital sodium injected through the penile vein. Then static P-V curves were recorded. After the thorax and diaphragm were opened, the tracheostomy catheter was connected to a pressure transducer (Validyne model DP 45-32, Validyne Engineering Co., Northridge, CA, USA) with a syringe attached to it, and pressures were recorded on a polygraph (Grass model 7B, Grass Instrument Co., Quincy, MA, USA). Using a syringe filled with nitrogen (N₂) the lungs were first inflated (within 10 sec) to an airway pressure of 35 cm H₂O, which was maintained for 5 sec, followed by deflation to an airway pressure of 0 cm H₂O. Then the lungs were re-inflated in steps of 0.5 ml until an airway pressure of 35 cm H₂O was reached. Each inflation step took 1-2 sec followed by a 5-sec pause to allow pressure equilibration. After this, in the same way, the lungs were then deflated until an airway pressure of 0 cm H₂O was reached. The volume of N₂ left in the syringe

was recorded. The lower inflection point (LIP) was determined from the intersection of the lines representing the minimum slope of the compliance curve and the maximum slope of the compliance curve. Maximal compliance (C_{\max}) was calculated from the steepest part of the deflation limb ¹⁹. Total lung capacity (TLC_{35}) was defined as lung volume at inflation with a distending pressure of 35 cm H₂O ²⁰.

Gruenwald Index

The Gruenwald index which characterises the surfactant system *in situ* ²¹, was calculated from the P-V curve, defined as $(2V_5+V_{10})/2V_{\max}$, where V_5 , V_{10} and V_{\max} are the lung volumes at transpulmonary pressures of 5, 10 and 35 cm H₂O from the deflation limb, respectively.

Bronchoalveolar lavage (BAL)

After the P-V curve recordings a BAL (30 ml/kg) was performed five times with saline-CaCl₂ 1.5 mmol/l (crude lavage). Thereafter, cell debris were removed from BAL by centrifugation at 400 g for 10 min. The active surfactant component in the BAL fluid was separated from the non-active surfactant component by differential centrifugation, followed by phosphorus analysis, and the ratio of non-active to active (small to large aggregate) surfactant was calculated ²². Finally, the protein concentration of the BAL fluid was determined using the Bradford method (Bio-Rad protein-assay, Munich, Germany) ²³.

Minimal Surface Tension

Minimal surface tension of the crude lavage was determined by means of a modified Wilhelmy balance (E. Biegler GmbH, Mauerbach, Austria). In this method, a tight-fitting teflon barrier reduces the surface area of a teflon trough from 100-20% at a cycle speed of 0.33/min. Saline is used as subphase and is kept at 37°C. The force on a platinum slide (1x1 cm) is measured by a force transducer and expressed as surface tension. Further, maximal surface tension is measured at 100% surface area and minimal surface tension at 80% surface compression and expressed as milli Newton/meter (mN/m). Surface tension characteristics of a BAL sample are measured after application on the surface of the saline-filled trough. In this study 300 µl of BAL fluid was applied to the surface of the trough; surface tension was measured after 3 cycles ²⁴.

Statistical analysis

Statistical analysis was performed using the Instat 2.0 biostatistics package (GraphPad software, San Diego, CA, USA). Intragroup comparisons were analysed with repeated measures ANOVA; intergroup comparisons were analysed with ANOVA. If a difference was found, a post-hoc test was performed (Tukey-Kramer). Statistical significance was accepted at p-values <0.05. All data are expressed as mean \pm standard deviation.

Results

Figure 1 shows the PaO₂ levels during the whole study period. After VILI and after the ventilator settings were set at 26/6 cm H₂O for 5 minutes the PaO₂ decreased below 13.3 kPa in all groups. After PFC instillation and after increasing the pressures to 30/10 cm H₂O the PFC₂₀ and PFC_{20+R} groups showed a significant increase in PaO₂ values to pre-VILI levels (p < 0.001), but only the PFC_{20+R} group maintained oxygen tension levels above 60 kPa during the 2-h study period. In both groups with a single dose of PFC (PFC₁₀ and PFC₂₀) PaO₂ values decreased over time. There were significant differences between the values in the Ventilated and PFC₁₀ groups compared with the values of the PFC_{20+R} throughout the study period (p < 0.001).

Table 1 shows that the PaCO₂ values and MAP levels were comparable in all groups during the whole study period.

Table 2 shows data from BAL fluid and lung mechanics. Protein concentration was significantly higher in the PFC₁₀ group compared with the Non-Ventilated group. The Gruenwald Index and the minimal surface tension of the crude lavage fluid, from all ventilated groups were not significantly different from the Non-Ventilated control group. For data on TLC₃₅, C_{max}, and LIP, see Table 2 and Fig. 2a/b. The total phosphorous concentration in the BAL fluid was not different between groups.

The ratio of small to large aggregates in BAL fluid was significantly higher in the four ventilated groups compared with the Non-Ventilated control group, and there was no significant difference between the ventilated control group and all the PFC-treated groups.

Figure 2a shows the inflation limbs from the P-V curves. Both PFC treated groups with 20 ml/kg have a significantly lower opening pressure (LIP) than both Non-Ventilated and the Ventilated control groups. Figure 2b shows the deflation limbs from the P-V curves. The three PFC treated groups had a significantly higher TLC₃₅ and C_{max} than both the Non-Ventilated group and the Ventilated control groups (Table 2).

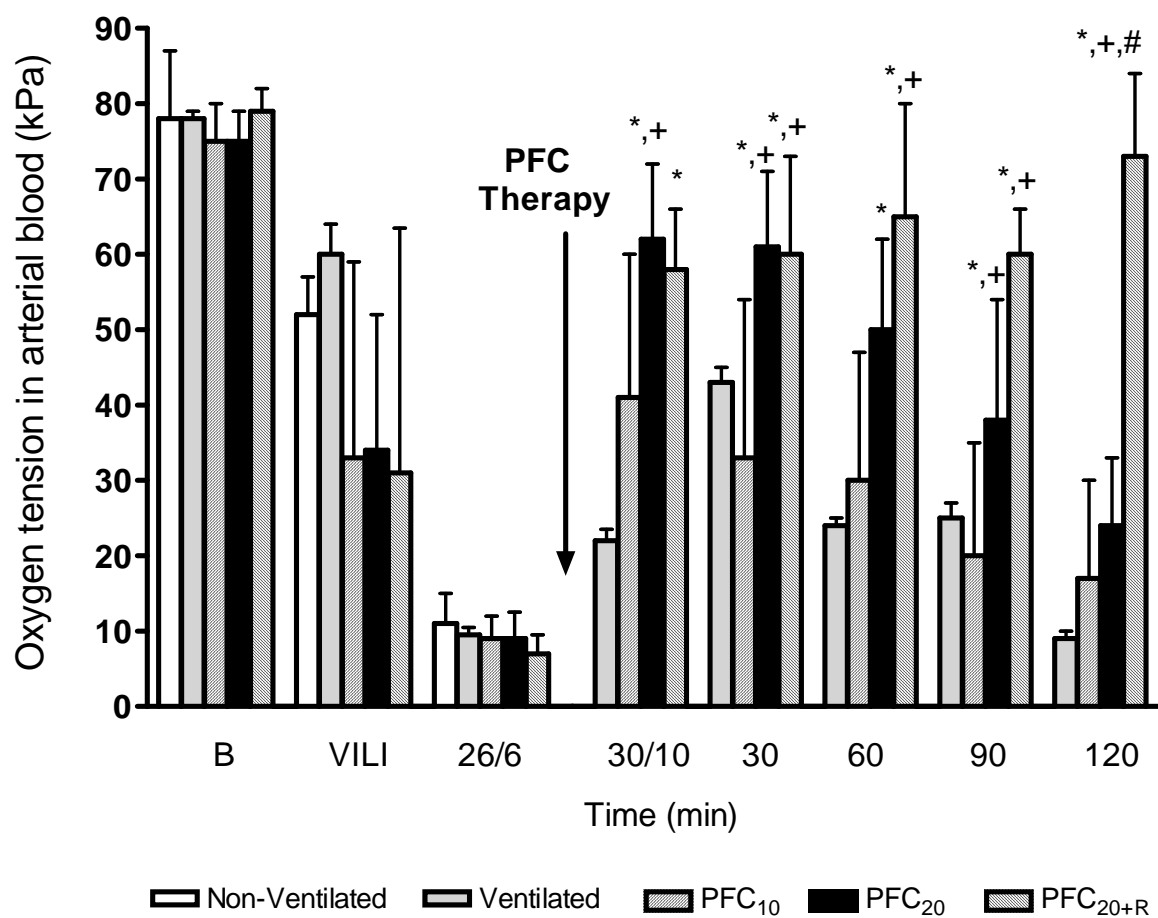


Figure 1. Arterial oxygen tension (mean \pm standard deviation) during the whole study period. B = baseline, VILI = ventilation with Peak inspiratory pressure (PIP) of 45 cm H₂O without PEEP after 20 min, 26/6 = after 5 min at PIP 26 cm H₂O, 6 cm H₂O PEEP. * indicates significant difference with Ventilated control group, + indicates significant difference with PFC₁₀, # indicates significant difference with PFC₂₀.

Table 1. Data on arterial carbon dioxide tension (PaCO₂) and mean arterial pressure (MAP) over time

	Time	Non-Ventilated	Ventilated	PFC ₁₀	PFC ₂₀	PFC _{20+R}
PaCO₂ (kPa)	Baseline	4.7±0.7	5.3±1.6	4.8±0.97	4.9±0.8	5.6±1.7
	VILI	2.5±0.3	2.4±0.6	2.5±0.35	2.7±0.4	2.9±0.5
	5' 26/6	4.7±0.7	5.3±1.0	5.0±2.1	5.2±0.89	6.7±1.7
	5' 30/10		5.3±1.3	4.3±0.4	4.8±0.7	5.7±0.8
	30'		5.6±1.3	3.9±0.4	4.4±0.9	5.4±0.9
	60'		6.3±1.6	4.4±1.3	4.4±0.7	4.7±1
	90'		6.5±1.3	4.8±1.2	4.7±0.5	5.6±0.8
	120'		6.9±1.8	4.8±2.1	4.9±0.3	4.4±0.9
	MAP (mmHg)	Baseline	135±16	134±15	144±6	140±24
VILI		75±25	84±32	72±25	85±22	71±21
5' 26/6		74±30	63±42	47±24	67±36	89±32
5' 30/10			111±12	88±24	106±25	97±14
30'			91±24	80±20	89±21	90±9
60'			89±19	78±17	95±15	95±37
90'			86±29	76±17	97±15	97±12
120'			89±27	85±9	73±24	80±13

Non-Ventilated = Non-ventilated control. Ventilated = Ventilated control. PFC₁₀ = partial liquid ventilation (PLV) with perfluorocarbons (PFC) at dose 10 mg/kg, PFC₂₀ = PLV with PFC at dose 20 mg/kg; PFC_{20+R} = PLV with PFC at dose 20 mg/kg plus 5 mg/kg after 60 minutes of ventilation. VILI = ventilation-induced lung injury, Baseline = measurement before VILI. Values are mean ± standard deviation.

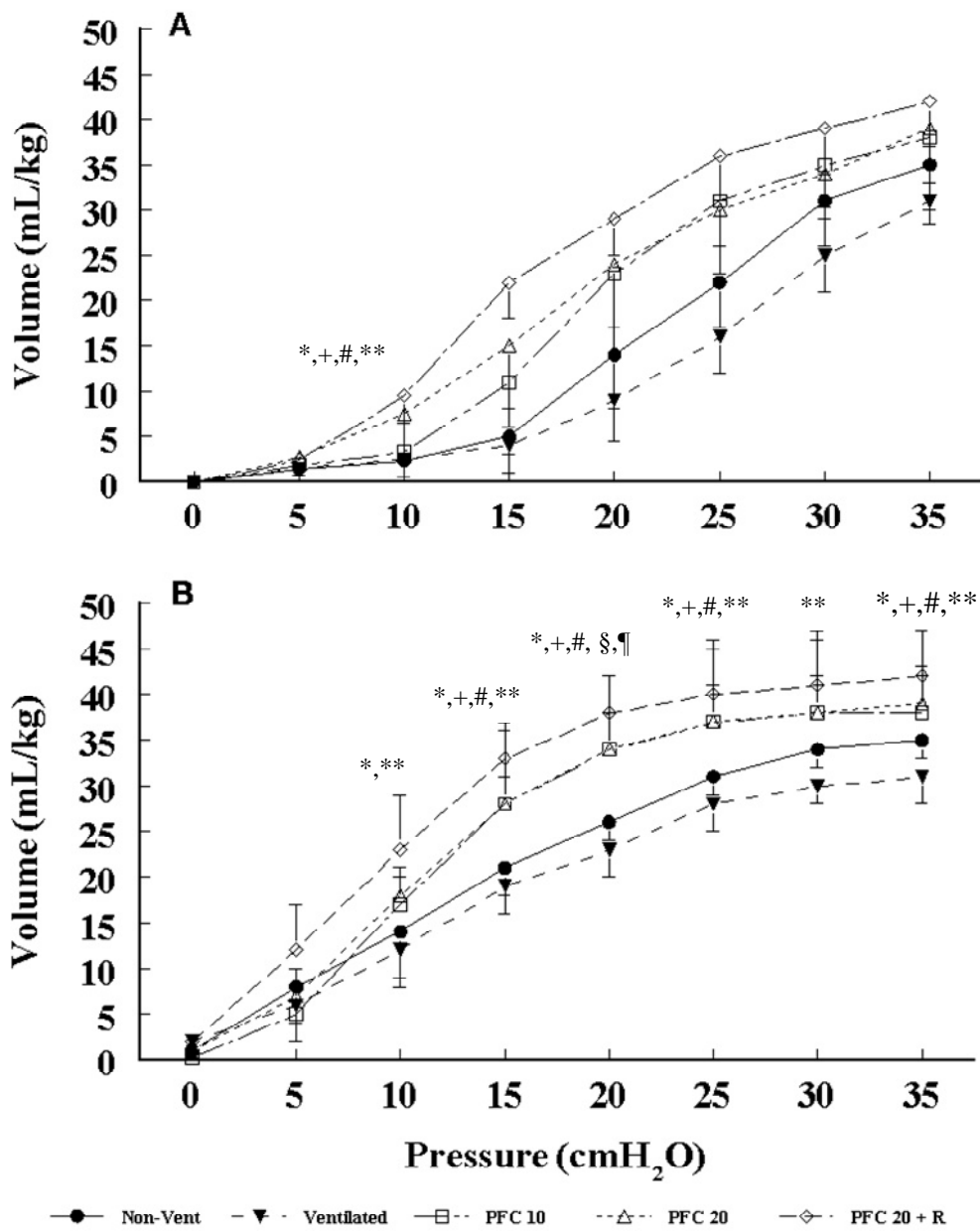


Figure 2. 2.a) Inflation limbs from the pressure-volume curves, (mean \pm standard deviation). Non-Vent = Non-ventilated group. Lower inflection point. 2.b) Deflation limbs from the pressure-volume curves, (mean \pm standard deviation). * indicates significant differences PFC_{20+R} vs Non-Ventilated, ** indicates significant differences between PFC_{20+R} vs Ventilated, + indicates significant differences between PFC₂₀ vs Ventilated, # PFC₁₀ vs Non-Ventilated, ¶ indicates significant difference between PFC₁₀ vs Ventilated, § indicates significant difference between PFC₂₀ vs Non-Ventilated.

Table 2. Amount of recovered broncho-alveolar lavage (BAL) fluid, protein concentration, lung volume above FRC at pressure 35 cm H₂O (TLC₃₅), maximum compliance (C_{max}), Gruenwald Index, lower inflection point of the pressure-volume curve (LIP), minimal surface tension (min surf) of crude BAL fluid, total phosphorus concentration, and small aggregates (SA)/large aggregates (LA) ratio.

	Non-Ventilated	Ventilated	PFC ₁₀	PFC ₂₀	PFC _{20+R}
Recovery BAL fluid (%)	90±1	90±1	90±1	90±1	90±1
Prot. Conc. BAL (mg/ml)	1.3±0.3*	1.4±0.4	1.9±0.2	1.8±0.3	1.4±0.1*
TLC₃₅	35±2**	31±3*+**	38±4	39±4	42±5
C_{max} (ml/kg)	1.5 ±0.3*+**	1.4±0.2*+**	2.4±0.2	2.6±0.2	2.8±0.6
Gruenwald index	0.30±0.09	0.40±0.17	0.40±0.07	0.4±0.07	0.5±0.2
LIP (cm H₂O)	15.3±1.4*+**	18.2±2*+**	10.8±1.2**	10.6±3.3**	6.7±1.9*
Min surf (mN/m)	33±3.1	31±1.6	35±2.1	35±0.5	32±3.9
Total phosphorus (mmol)	2.0±0.6	1.4±0.5	1.7±0.4	1.5±0.3	1.5±0.2
SA/LA ratio	1.7±1.1	4.6±2.7	4.6±2.3	5.6±3.5	4.2±2.7

Non-Ventilated = Non-Ventilated Control group. Ventilated = Ventilated control group. PFC₁₀ = PFC 10 mg/kg. PFC₂₀ = PFC 20 mg/kg. PFC_{20+R} = PFC 20 mg/kg + 5 mg/kg at 60 min of study period to replace PFC loss due to evaporation. Values are mean ± standard deviation. * p<0.05 vs PFC₁₀, + p<0.05 vs PFC₂₀, ** p<0.05 vs PFC_{20+R}

Discussion

This study shows that partial liquid ventilation improves PaO₂ and lung mechanics in ventilation-induced lung injury, despite the presence of a high intra-alveolar protein concentration.

In the present study, 20 min of ventilation with high peak-inspiratory pressures without PEEP resulted in pulmonary edema and hypoxemia, and in impairment of the pulmonary surfactant system. The latter is characterised by a decrease in lung mechanics and Gruenwald Index, and a high minimal surface tension in BAL fluid compared with healthy rat lungs [10](#). The exact mechanism by which the lung damage is produced by artificial ventilation is not yet entirely clear, but the role of surfactant changes is becoming increasingly realised [10, 11, 17](#). Recently, our group showed that modes of mechanical ventilation with large tidal volume without PEEP disturb the surfactant system in the used animal model of VILI [10](#). It has been demonstrated that loss of surface active molecules due to mechanical ventilation with high inspiratory lung volumes

without PEEP is produced by displacement of surfactant from the alveolar air-liquid interface into the small airways ^{4, 7}. Moreover, the surface area changes produced by the high inspiratory lung volumes lead to an increased rate of conversion of active into non-active surfactant subfractions ⁸⁻¹¹. These together will lead to alveolar collapse and protein infiltration ^{4-6, 10, 11} in which the latter leads to further inactivation of surfactant ^{25, 26}. In the current study we used partial liquid ventilation to correct the lung function affected by VILI. The results shown that after VILI partial liquid ventilation produced an immediate improvement in PaO₂, dose and time dependent. In the group treated with 10 ml/kg of perfluorocarbon the pre-VILI values of PaO₂ were never reached, while in both groups treated with 20 ml/kg PFC within 5 min there was a significant increase in PaO₂ values compared with values after VILI, and these improved values were comparable with baseline values. However, PaO₂ decreased over time in both groups in which perfluorocarbon was not replaced. It has been shown that in surfactant-deficient animal lungs partial liquid ventilation provides adequate gas exchange as long as a sufficient amount of PFC is present in the lungs ¹²⁻¹⁵. Our group has demonstrated that higher doses of PFC lead to higher levels of oxygenation in animals suffering from acute respiratory failure as a result of dose-dependent recruitment of collapsed atelectatic alveoli by PFC ^{12, 13}. It is also known that oxygenation deteriorates over time if no additional doses of PFC are instilled; this is attributed to evaporation of PFC which will cause affected alveoli to collapse ^{12, 13}. In the present study, the inflation limbs of the P-V curves showed on the one hand a significantly lower opening pressure in the three PFC-treated groups, and on the other a significantly higher total lung capacity and maximal compliance compared with both control groups. The reason for this is that in surfactant-deficient lungs, the decrease of surface tension at the air-liquid interface by PFC improves the mechanical properties of the lung ^{12, 13, 27-30}. Dreyfuss et al. advocated that an important benefit of PFC on lung mechanics was the reduction of the mechanical nonuniformity of flooded lungs and probably opposition to overinflation of the more compliant, aerated zones ²⁷. But the results from the present study and other studies ^{29, 30} would not support the findings of Dreyfuss and colleagues because our data, which characterise (indirectly) overinflation of alveoli i.e. a high SA/LA ratio ^{8, 9} and high alveolar protein influx ⁵, just indicate overinflation.

A side effect of PFC may be the constant surface tension which does not change with the changes in surface area (which is a property of natural surfactant) so that the end-expiratory stability in the PFC-treated animals (characterised by the Gruenwald Index) was the same as in the Ventilated control animals ¹⁵.

As mentioned above, in the current study the protein level from the crude BAL fluid in all ventilated groups was as high as in the Non-Ventilated control group. Moreover, in both PFC treated groups without replacement, an increase in the total protein concentration was observed. Dreyfuss et al. showed in rats that PFC partially reversed the effects of alveolar flooding, but did not reduce the permeability changes on the alveolo-capillary membrane measured by ¹²⁵I-labeled serum albumin [27](#). This is partially supported by our data showing that with a larger amount of PFC in the lung, the total amount of protein in the BAL fluid is less. How PFC prevents protein infiltration and alveolar flooding is not entirely clear. It has been suggested that as a result of the PFC-filled alveoli the suction forces on the interstitium more or less disappear thus preventing protein influx into the PFC-filled alveoli. However, alveoli which have only a PFC film at the air-liquid interface and which may collapse during expiration will promote alveolar flooding due to their high surface tension at end-expiration: this explains why the amount of proteins in the PFC group receiving only 10 ml/kg PFC is significantly higher than in the group treated with 20 ml/kg of PFC.

In conclusion, our results in this animal study show that, in VILI, partial liquid ventilation improves gas exchange and pulmonary function, despite the presence of a high intra-alveolar protein concentration. However, the loss of perfluorocarbon over time due to evaporation has to be replaced.

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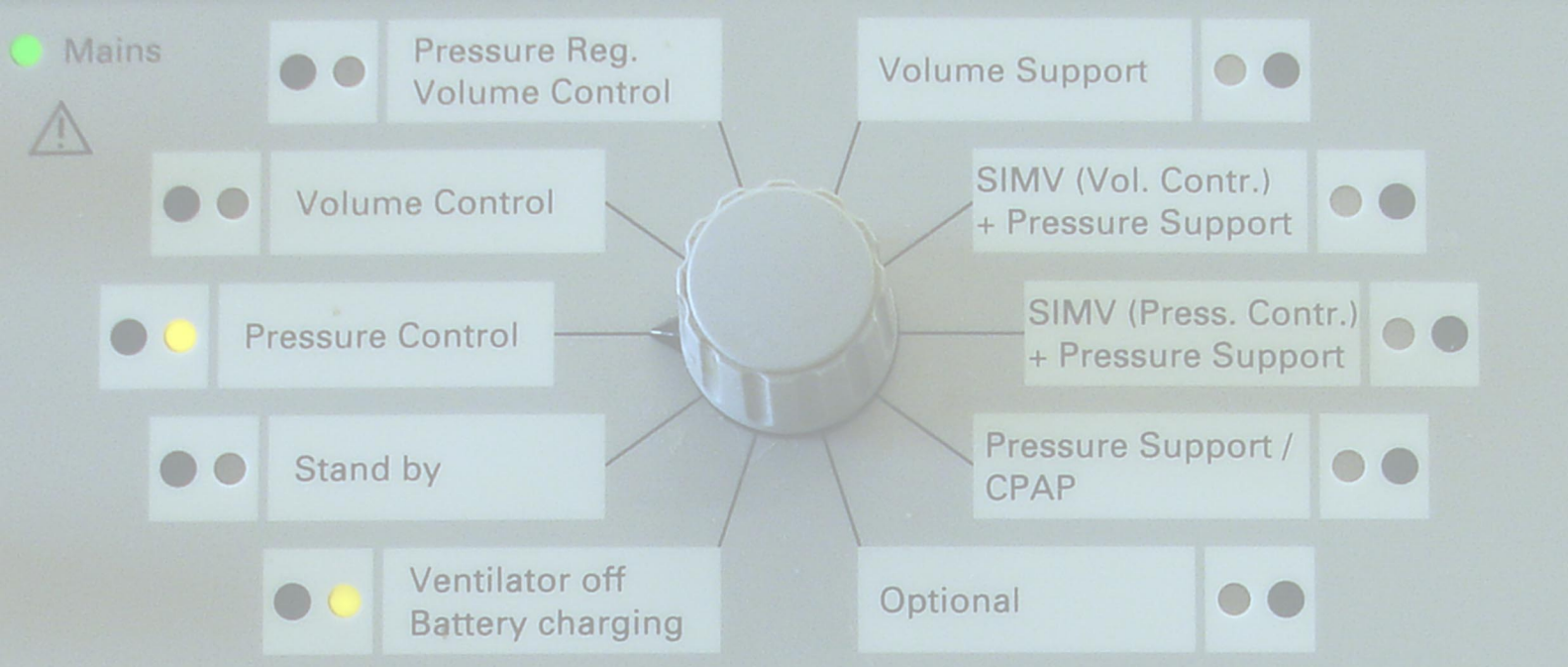
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Lot No: ZY23001

Vial 1



Resp. pattern

Measured freq.
b/min
50

Set freq.
b/min
50

Insp. period
s
0.40

Insp. flow
l/s

Chapter 10

Small-dose perfluorocarbon reduces the recruitment pressure needed to open surfactant-deficient atelectatic lungs

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Abstract

Background: To study the efficacy of a small dose of perfluorocarbon on the recruitment pressure needed to open atelectatic lung areas.

Methods: In 12 Yorkshire pigs (9 kg bodyweight) lung injury was induced by whole lung lavage. After one hour of conventional ventilation an open lung maneuver was performed to obtain PaO₂ values equal to the prelavage PaO₂ values ($\pm 10\%$). After one hour of ventilation at the lowest possible airway pressures that stabilized the recruited lung volume, the animals were disconnected from the ventilator to allow the lung to collapse. Six animals received a 5 ml kg⁻¹ intratracheal dose of perfluorocarbon and a second open lung maneuver was performed. Six animals served as controls and received no perfluorocarbon but also underwent a second open lung maneuver.

Results: In both groups an open lung maneuver resulted in a significant increase in oxygenation. Peak pressure needed to open the lung after 1 hour of mechanical ventilation in the perfluorocarbon group and in the control group was 43.8 ± 8.4 cmH₂O and 46.6 ± 4 cmH₂O, respectively. The addition of perfluorocarbon significantly reduced the opening pressure to 34.5 ± 6.3 cmH₂O ($p < 0.01$), whereas the opening pressure in the control group 45.0 ± 0.2 cmH₂O did not change.

Conclusion: Instillation of a small amount of perfluorocarbon significantly reduces the opening pressures needed to recruit atelectatic lung areas.

Introduction

Protective ventilation strategies are currently used in the treatment of injured lungs to prevent ventilator-induced lung injury (VILI) ¹⁻⁴. In these strategies, the beneficial effect of lower tidal volumes has been demonstrated in an ARDS trial in adults resulting in a reduction of mortality ². These ventilator settings help limit the ‘shear’ forces between open and collapsed alveoli, which are the predominant cause for VILI and mortality ³⁻⁵. To minimize VILI, an active recruitment procedure has been proposed which opens up atelectatic lung areas combined with ventilator settings that prevent alveolar collapse, resulting in prevention of shear forces and optimized ventilation ^{1, 3, 4, 6-8}. This recruitment procedure is performed by increasing peak pressures and concomitantly using a sufficiently high level of PEEP to stabilize these recruited alveoli. In severe ARDS, pressures as high as 60-70 cmH₂O may be required to recruit collapsed alveoli ^{6, 9-15}. However, during this maneuver a brief period of high mean airway pressure leads to alveolar overdistension of already open parts of the lung that can result in barotrauma and in haemodynamic instability.

Our group earlier reported that even a small dose of 5 ml kg⁻¹ perfluorocarbon (PFC) can reduce mean airway pressures in lung injury ¹⁶. We speculate that application of PFC could help to reduce the recruitment pressures needed to open up atelectatic lung areas in acute lung injury (ALI).

Therefore, in a well-defined model of ALI, we studied the efficacy of a small dose of PFC on the recruitment pressures needed to recruit atelectatic lung areas according to the open lung maneuver ¹⁷.

Materials and methods

Animal preparation

This study was approved by the local Animal Committee of the Erasmus University Rotterdam. Care and handling of the animals were in accordance with the European Community guidelines. Anesthesia was induced in 12 male Yorkshire pigs (bodyweight 9 ± 1 kg) with 10 mg kg⁻¹ ketamine (Ketalin 100 mg ml⁻¹, Apharmo, Arnhem, the Netherlands) and 0.5 mg kg⁻¹ midazolam (Dormicum 5.0 mg ml⁻¹, Roche Ned., Mijdrecht, the Netherlands) intramuscularly. After obtaining intravenous access a loading dose of 35 µg kg⁻¹ fentanyl (fentanyl 0.05 mg ml⁻¹, B. Braun Melsungen AG, Melsungen, Germany) was given and a continuous infusion of fentanyl (35 µg kg⁻¹ h⁻¹) and midazolam (9 µg kg⁻¹ h⁻¹) was started. After placing a 6-mm

endotracheal tube via a tracheotomy animals were connected to a ventilator (Siemens Servo 300, Solna, Sweden) set in a pressure regulated volume controlled mode (PRVC) with the following settings: frequency 20 min⁻¹, inspiratory time 33%, inspiratory rise time 0%, PEEP 5 cm H₂O and 100% oxygen. Minute ventilation was set to deliver a tidal volume (TV) of 10 ml kg⁻¹ BW. Muscle relaxation was achieved by continuous infusion of 2.5 µg kg⁻¹ min pancuronium bromide (Pavulon; Organon Technika, Boxtel, the Netherlands).

Subsequently, the carotid artery was cannulated to obtain arterial blood pressures and a blood gas monitoring sensor (Paratrend, Diametrics Medical Ltd, High Wycombe, England) was inserted to continuously analyze blood gases and pH. The right internal jugular vein was cannulated and a 5 Fr pulmonary artery catheter (TD catheter 4 lumen; Arrow Holland, Houten, the Netherlands) was introduced. Temperature was kept in normal range by a heating pad. Initial values (healthy) of all measured parameters were recorded.

Induction of lung injury

Prior to surfactant depletion all animals received an intravenous (i.v.) 20 ml kg⁻¹ bolus of a lactated Ringer's solution followed by a continuous i.v. drip of 10 ml kg⁻¹ h⁻¹. Lungs were surfactant depleted, as previously reported and originally based on a report by Lachmann et al. [18](#), by repeated whole lung lavage with warm saline (38°C, 30 ml kg⁻¹) to reduce PaO₂ below 13.3 kPa at the above-mentioned settings; with these ventilator settings animals were then ventilated for a 1-hour stabilisation period. After one hour of ventilation, baseline (Before-1) measurements were recorded.

Measurements

Gas exchange: Arterial blood gases and pH were continuously monitored with the Paratrend blood gas sensor (Paratrend, Diametrics Medical Ltd, High Wycombe, UK). However, for reference values, blood samples were taken and analysed in a conventional blood gas analyzer (ABL 505, Radiometer, Copenhagen, Denmark).

Haemodynamics: Using Statham P23XL transducers (Spectramed, Oxnard, CA, USA) mean arterial pressure (MAP) as well as mean pulmonary artery pressure (MPAP), pulmonary capillary wedge pressure (PCWP) and central venous pressure (CVP) were recorded in all animals. Cardiac output (CO) was measured in triplicate using the thermodilution technique with 5 ml cold saline, using a Sirecust 1280 monitor (Siemens, Danvers, MA, USA) that also recorded heart rate (HR).

Ventilatory parameters: Peak pressure was recorded from the ventilator and total end-expiratory pressure was measured using end-expiratory hold.

Open lung maneuver

We used arterial oxygen tension at 100% O₂ to characterize the presence of atelectasis, a PaO₂ > 66.7 kPa was defined as an open lung. To recruit collapsed lung tissue to the pre-lavage level an Open lung Maneuver (OLM) was performed. The open lung situation was defined as the point at which the PaO₂ value equaled the prelavage PaO₂ value within 10%. Then the ventilator was switched to pressure controlled ventilation set at: frequency 20 min⁻¹, inspiratory time 66%, inspiratory rise time 0%, PEEP 20 cm H₂O and 100% oxygen. Peak pressure was set to deliver a TV of 10 ml kg⁻¹ BW.

Peak inspiratory pressure was increased in 3 cmH₂O increments at 1-minute intervals until the PaO₂ value was equal to the prelavage PaO₂ value ($\pm 10\%$). This situation was recorded as pressure open (P_{open}). This procedure always resulted in hypocapnia and intrinsic PEEP.

Immediately after P_{open}, peak pressure was reduced to deliver TV < 7 ml kg⁻¹ BW. In order to find the minimal stabilizing pressure of the lung, at 1-minute intervals PEEP was decreased in 2 cmH₂O decrements, or when TV > 7 ml kg⁻¹ BW, peak pressure was decreased again to get a TV < 7 ml kg⁻¹ BW. The decrease in airway pressure was continued until the PaO₂ value decreased 10% below the prelavage PaO₂ value. This situation was recorded as pressure collapse (P_{collapse}). After P_{collapse} a re-opening procedure was performed using the ventilator settings recorded at P_{open} for 15 sec; subsequently, the ventilator was set at the settings obtained at P_{collapse} but PEEP was set 2 cmH₂O higher and peak pressure was adjusted to deliver TV of 5-6 ml kg⁻¹ BW. Settings were left unchanged for 1 hour followed by renewed measurements of the parameters, which were recorded as P stable (P_{stable}). P_{stable} was defined as the ventilator settings which resulted in stable PaO₂ and PaCO₂ values over time.

After recording P_{stable}, to allow the lung to collapse the ventilator was disconnected for 30 sec from the endotracheal tube. Then, the ventilator was set to the 'lavage values': PRVC, frequency 20 min⁻¹, inspiratory time 33%, inspiratory rise time 0%, PEEP 5 cm H₂O and 100% oxygen. Minute ventilation was set to deliver a TV of 10 ml kg⁻¹ BW. A second baseline measurement was recorded 5 min after these new settings. Animals were then randomized to receive either 5 ml kg⁻¹ PFC (APF-175A, perfluorodimethyldecalin, FluoroMed, L.P. Inc Round Rock, TX, USA) (n=6) or to serve as a control (n=6). After 5 min the open lung procedure was then performed again as described above. The design of the study is shown in Figure 1.

At the end of the study period all animals were killed with an intracardiac overdose of KCl.

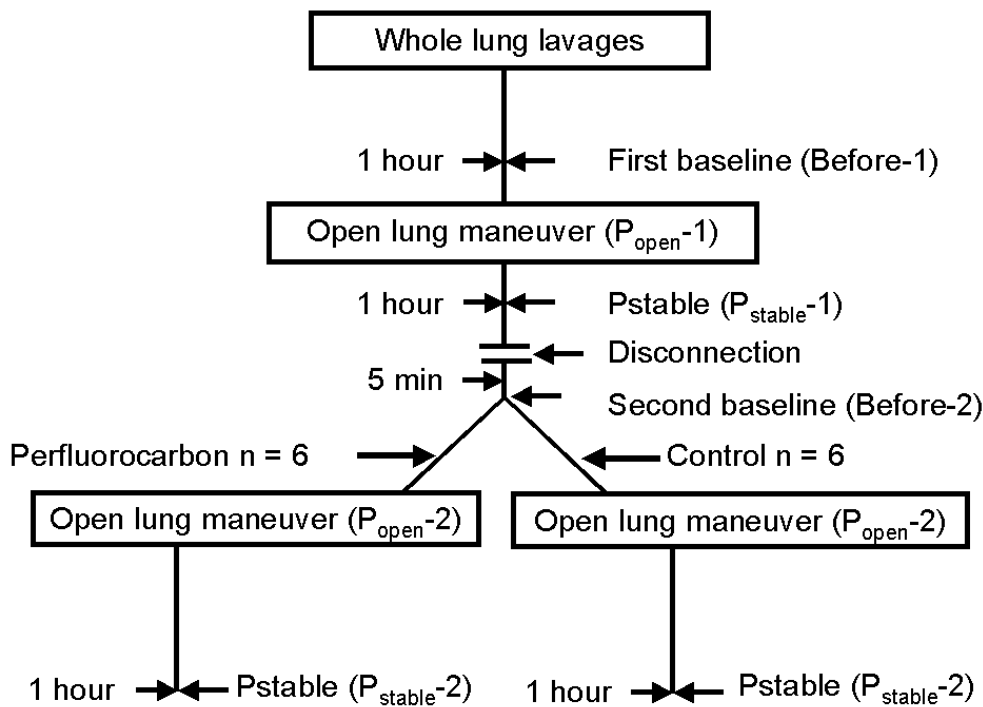


Figure 1. Diagram showing the interventions and measurements made in the present study.

Statistical analysis

Data analysis was performed using the Instat 2.0 biostatistics package (GraphPad Software, San Diego, USA). For all values obtained during each point of the open lung maneuver intra-group comparisons were made with repeated measures ANOVA. If ANOVA resulted in a $p < 0.05$ a Dunnett post test was performed. All data are reported as mean values \pm standard deviation (SD), and $p < 0.05$ was considered significant.

Results

The weight of the animals was 9.4 ± 0.4 kg. All animals survived the study period. There were no pneumothoraxes in any of the animals. Oxygenation was above 66.7 kPa ($p < 0.001$) in all animals after the Open Lung Maneuver (OLM) was performed compared to the oxygenation level of the collapsed lung (Fig. 2).

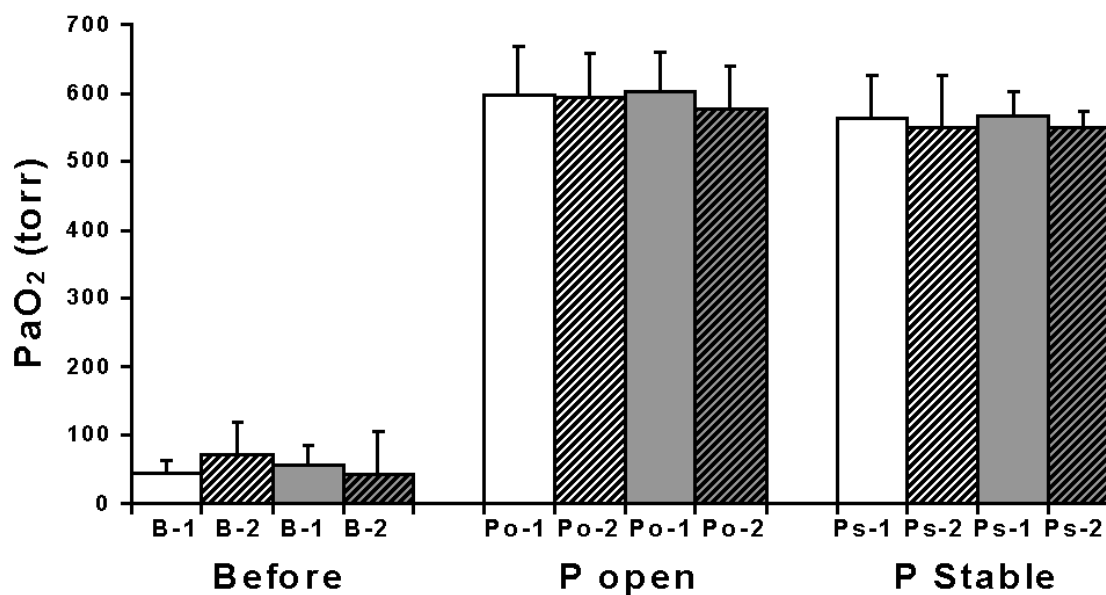


Figure 2. Arterial oxygenation 1 hour after induction of lung injury (Before), at maximal alveolar recruitment (P_{open}) and at the lowest airway pressures that maintain optimal oxygenation for 1 hour (P_{stable}). Study group animals before (open bars) and after application of perfluorocarbon (white/black diagonal striped bars). Control group animals: first recruitment (gray bars) and second recruitment (gray/black diagonal striped bars). Data are mean \pm SD.

No significant difference in oxygenation was observed at any time between the two groups, or between P_{open} values and after 1 hour of ventilation P_{stable} . During the OLM there was a significant decrease in $PaCO_2$, during the subsequent 1-hour ventilation period all animals were normocarbic (Table 1). Peak pressure needed to open the lung after 1 hour of lung injury was 43.8 ± 8.4 cmH₂O. The addition of PFC significantly reduced the opening pressure by almost 10 cmH₂O ($p < 0.01$), whereas the opening pressure in the control group did not change (Fig. 3). However, the pressure to stabilize (P_{stable}) the opened lung for 1 hour after P_{open} did not differ between both groups (Fig. 3). Although MAP decreased in both groups during the opening maneuver, this was only significant in the second opening maneuver in the control group (P_{open-2}) ($p < 0.05$), whereas there was no significant difference in the PFC group (Table 1). Decrease of the airway pressures to P_{stable} values restored MAP to pre-opening values (Table 1). In both groups there was a significant increase in PCWP during the opening procedures, CVP was increased and CO decreased although this was not significant (Table 1).

Table 1. Data on PaCO₂, arterial pH, and hemodynamic parameters.

PFC							
	Healthy	Before-1	P _{open} -1	P _{stable} -1	Before-2	P _{open} -2	P _{stable} -2
PaCO ₂	4.1 (0.6)	6.5 (2.0)	3.7(0.6)	4.9 (0.4)	7.2 (1.4)	4.5 (1.0)	4.7 (0.3)
PH	7.53 (0.05)	7.32 ^a (0.18)	7.44 (0.1)	7.35 (0.07)	7.22 (0.12)	7.36 (0.12)	7.38 (0.05)
MAP	89 (10)	89 (19)	76 (19)	95 (21)	100 (16)	84 (25)	102 (9)
MPAP	32.3 (8.6)	35.5 (7.5)	36.7 (5.0)	35.7 (6.3)	47 (7.2)	34.7 ^a (3.	34.2
CO	2.2 (0.9)	2.0 (0.6)	1.0 ^a (0.3)	1.0 (0.2)	1.5 (0.4)	0.9 (0.5)	1.0 (0.3)
CVP	7 (2)	7 (1)	13 (4)	9 (5)	9 (4)	11 (5)	10 (5)
PCWP	11 (3)	11 (3)	23 ^c (7)	15 ^b (4)	13 (4)	19 ^b (5)	15 ^d (5)
HR	132 (30)	140 (22)	159 (40)	154 (30)	169 (21)	177 (31)	146 (32)
Control							
PaCO ₂	3.8 (0.5)	5.4 (1.3)	3.1 (0.8)	4.5 (0.6)	6.3 (1.1)	3.8 (0.6)	4.6 (0.6)
PH	7.55 (0.09)	7.43 (0.11)	7.55 (0.14)	7.41 (0.06)	7.36 (0.14)	7.47 (0.08)	7.40
MAP	95 (10)	97 (17)	87 (28)	105 (28)	124 (40)	76 ^a (5)	99 (21)
MPAP	26 (3)	28 (2)	32 (2)	35 (3)	48 ^b (9)	33 ^a (4)	34 (1)
CO	1.4 (0.4)	1.7 (0.7)	1.1 (0.4)	0.8 (0.3)	1.7 (0.9)	0.9 (0.2)	0.8 (0.3)
CVP	9 (2)	7 (1)	15 (5)	9 (2)	12 (9)	18 (11)	11 (1)
PCWP	13 (3)	8 (2)	21 ^c (5)	12 ^b (2)	13 (3)	22 ^a (2)	24 (5)
HR	138 (27)	119 (20)	139 (42)	142 (43)	142 (21)	154 (41)	156 (31)

PaCO₂, arterial pH, and hemodynamic values before lung injury (healthy) and after lung collapse (before), during the alveolar recruitment procedure (P_{open}) and after 1 h of ventilation at the lowest mean airway pressures (P_{stable}). In a low dose intratracheal administered perfluorocarbon group and a control group. The additive (-1) indicates the first and the additive (-2) indicates the second recruitment procedure. PaCO₂ (kPa); pH (arterial); MAP=mean arterial pressure (mmHg); MPAP=mean pulmonary artery pressure (mmHg); CO=cardiac output (l min⁻¹); CVP=central venous pressure (mmHg); PCWP=pulmonary capillary wedge pressure (mmHg); HR=heart rate (beats min⁻¹). Values are mean (SD).

^ap < 0.05, ^bp < 0.01, ^cp < 0.001 vs previous measurement and ^dp < 0.05 vs control group.

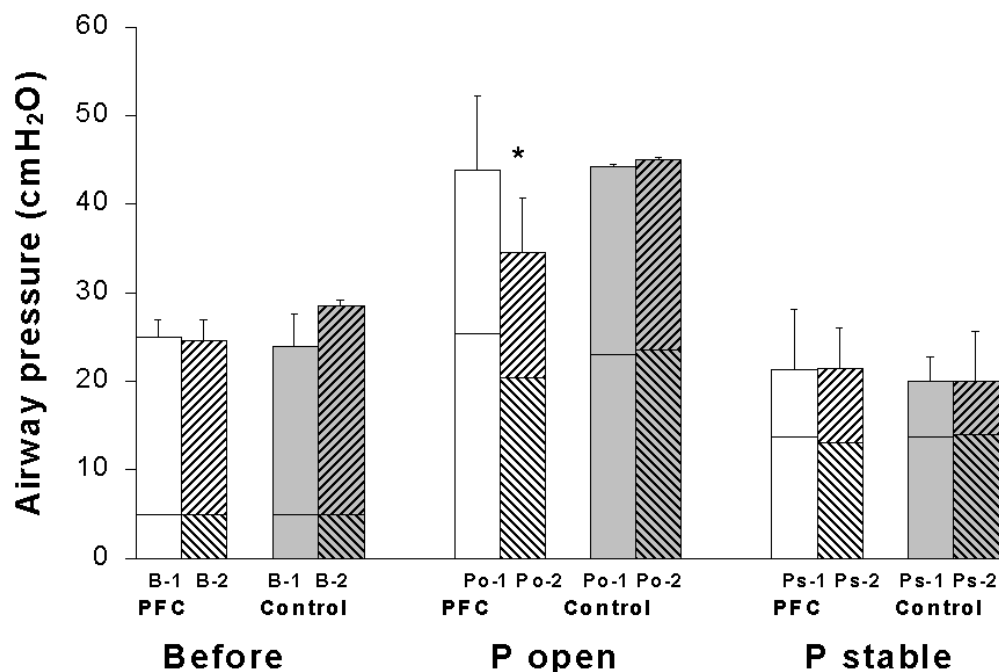


Figure 3. Airway pressures peak (top) and PEEP (horizontal line) after induction of lung injury (Before), at maximal alveolar recruitment (P_{open}) and at the lowest airway pressures that maintain optimal oxygenation for 1 hour (P_{stable}). Study group animals before (open bars) and after (white/black diagonal striped bars) application of perfluorocarbon. Control group animals: first recruitment (gray bars) and second recruitment (gray/black diagonal striped bars). Data are mean \pm SD. * $p < 0.05$ vs P_{open} in the PFC group.

There was an immediate increase in MPAP in both groups after disconnection from the ventilator (Before-2) probably due to atelectasis (hypoxic pulmonary vasoconstriction) (Table 1), which was resolved by the recruitment maneuver (P_{open-2}) ($p < 0.05$). During the following 1-hour observation period MPAP remained stable at this lower value (Table 1).

Discussion

Depending on the clinical condition, alveolar recruitment may require pressures ranging from 40 cmH₂O in healthy lungs to high pressures of 50 cmH₂O and above in ARDS and in hypoplastic lungs in the paediatric age group [6](#), [9](#), [11](#), [12](#), [14](#), [15](#). The present study demonstrates that an active recruitment maneuver results in low ‘shunt’ perfusion characterized by a $PaO_2 > 66.7$ kPa at 100% O₂.

When a low dose of PFC was added the pressure needed to open up the lung was significantly lower while oxygenation was comparable to lungs without the addition of PFC. An amount of 5

ml kg⁻¹ PFC administered intratracheally does not by itself substantially improve oxygenation, but does lower the mean airway pressure ¹⁹. This can be explained by the higher viscosity of the used PFC which had been shown to improve alveolar distribution ²⁰ and may also be due to the evaporation of PFC (vapour pressure of APF 175 A is 0.089 kPa at 25°C) and the subsequent formation of a thin layer of PFC in alveoli. This layer of PFC reduces surface tension (surface tension of APF 175 A is 20.5 mN/m), and according to the law of Laplace ($P = 2\gamma r^{-1}$; P = pressure in the bubble; γ = surface tension; r = radius of the bubble), which will allow opening of alveoli at lower peak airway pressures. However, due to the surface tension properties of PFC, on theoretical basis, the end-expiratory pressure needed to maintain an open lung would be higher. The results of this study did not show this effect. The reason for this could be that the applied strategy to keep the whole lung open, already required a PEEP level which was above the level required according to the surface tension of the used PFCs. The surface tension of the PFC used in this study (20.5 mN/m) is higher than the surface tension when active surfactant is present in healthy lungs, minimal surface tension lower than 5 mN/m during end expiration thus preventing end-expiratory collapse according to the law of Laplace. If the surface tension is higher this would result in end-expiratory collapse and one would have to compensate for the higher retraction forces due to increased surface tension (20.5 mN/m) by applying sufficiently high PEEP levels.

In a recent report by Cox and co-workers they showed similar results; a low dose of PFCs increased recruitment at the same mean airway pressure during high frequency oscillation ventilation compared to non-treated animals ^{21, 22}. Recruitment in this study was assessed by CT-scan ²¹. In contrast to this latter study, in each animal and after each intervention we established the opening pressure, stable pressure and the pressure where the lung collapsed. We clearly demonstrate that the addition of PFCs reduced the opening pressure.

Shunt is minimized by an active recruitment method, combined with a sufficiently high level of PEEP to avoid collapse of the recruited lung tissue. Recruitment of collapsed alveoli has been shown to improve oxygenation both in healthy ^{23, 24} and diseased lungs ^{3, 10, 11} and minimize shear forces, thus preventing lung injury ⁷. However, application of insufficient levels of PEEP, e.g. below the critical closing point during the recruitment maneuver, will result in re-formation of atelectasis ¹⁰. In this study, PEEP levels were sufficient, as indicated by stable PaO₂ levels during the 1-hour observation period after the opening maneuver.

In the present study, fluid management was limited to a single dose of 20 ml kg⁻¹ of lactated Ringer's in order to prevent obscuring of deleterious haemodynamic effects. Although it has been reported that high PEEP levels can reduce cardiac output ²⁵, a recent report indicated that

during an open lung maneuver haemodynamics are not affected ²⁶ furthermore adequate hydration can be used to maintain cardiac output ²⁷.

Surprisingly, we observed an increase in MPAP after disconnection and subsequent collapse of the lung (Table 1). Pulmonary hypertension is a common finding in ARDS ²⁸. An active recruitment procedure rapidly decreases the pulmonary artery pressure (Table 1), demonstrating that besides established interventions such as NO ²⁹ and prostacyclins, overcoming hypoxic-induced pulmonary vasoconstriction by recruiting lung tissue is a cost-effective alternative.

Patients with severe lung injury often require drugs to maintain adequate blood pressures, and even a short period of hypotension could result in complications in these patients. A recent study in which high-frequency oscillatory ventilation was combined with recruitment maneuvers showed that recruitment maneuvers failed due to hypotension in 6 out of 8 cases (in the other 2 cases no apparent reason was indicated) ³⁰. With this simple technique of applying a small amount of PFC we can significantly reduce the required opening pressure and thus reduce the decrease in blood pressure while optimizing arterial oxygenation and minimizing intrapulmonary shunt flow. Application of the open lung concept in both healthy lungs ²⁶ and in severe ARDS after pulmonary contusion ³¹ has shown to improve oxygenation and reduce the amount of atelectasis. The present study was performed in a single model of ARDS and other models of ARDS should be investigated before implementation in patients.

The endogenous surfactant system can be influenced by PFCs ³²; although in severe lung injury the surfactant system is already severely damaged and unable to maintain alveolar stability, the small amount of PFC used in the present study will probably not result in any additional changes in the surfactant system ³³. Although, up to now clinical studies have not been able to demonstrate a beneficial effect of partial liquid ventilation, the type of application presented in this study could be an indication for the future use of perfluorocarbons.

Conclusions

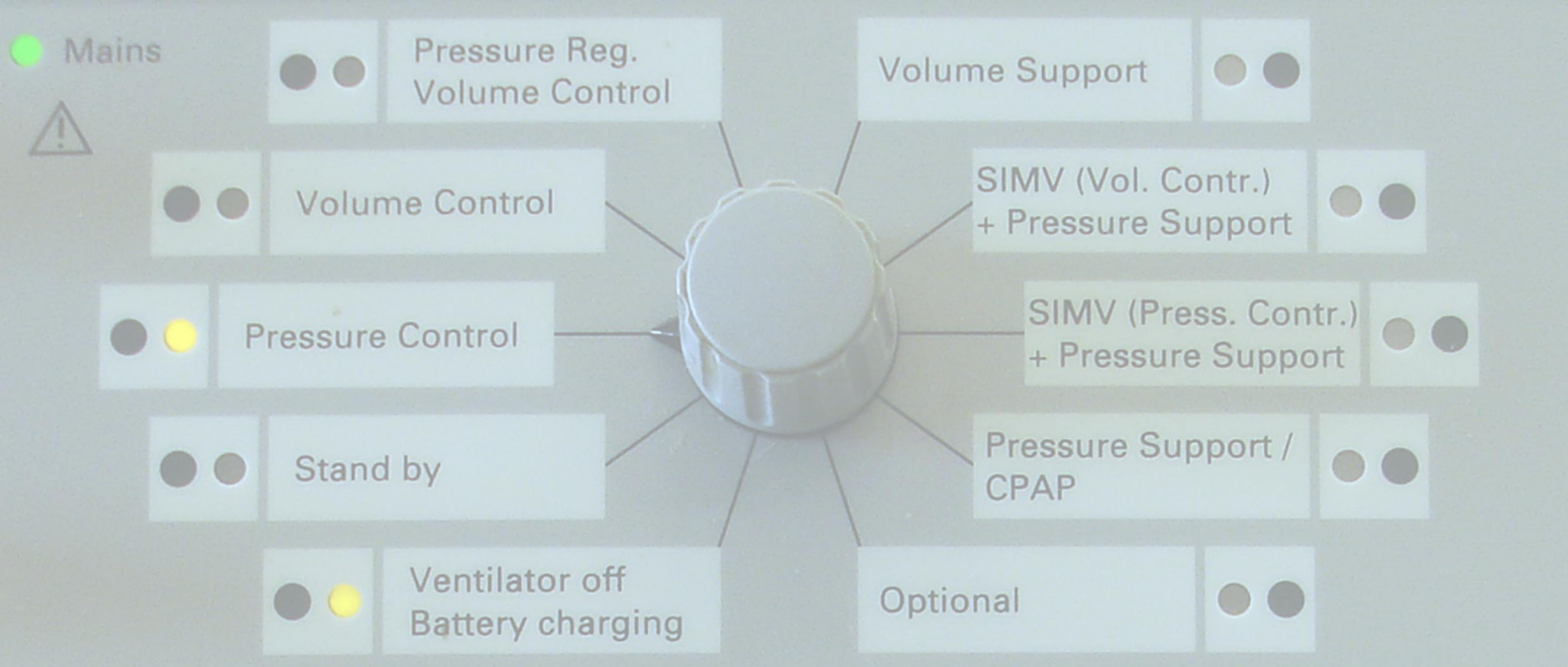
Active recruitment of atelectatic lung areas improves oxygenation and allows a reduction of airway pressures to stabilize the lung. Instillation of a small amount of perfluorocarbon significantly reduces the opening pressures needed to recruit atelectatic lung areas. Furthermore, hypoxic pulmonary vasoconstriction induced by atelectasis was immediately overcome by the recruitment procedure.

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Resp. pattern

Measured freq. b/min
50

Set freq. b/min
50

Insp. period s
0.40

Insp. flow l/s

Chapter 11

Treatment of ventilation-induced lung injury with exogenous surfactant

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Abstract

Pulmonary surfactant plays a role in ventilation-induced lung injury (VILI). Therefore, we investigated whether exogenous surfactant might restore gas exchange and lung mechanics in an established model of VILI. From 24 adult rats, 6 animals were killed immediately after induction of anesthesia and were used as healthy controls. In 18 rats, VILI was induced by increasing peak inspiratory pressure (PIP) to 45 cm H₂O without positive end-expiratory pressure (PEEP) for 20 min. Thereafter, animals were randomly divided into three groups of six animals each: One group was killed immediately after VILI (VILI-control). In the other two groups, ventilator settings were changed to PIP of 30 cm H₂O and PEEP of 10 cm H₂O, and respiratory rate of 40 bpm. One group received surfactant and the other group received no treatment. Blood gas tension and arterial blood pressures were recorded every 30 min for two hours. Then, a pressure-volume curve was recorded, a broncho-alveolar lavage was performed to determine protein content, minimal surface tension and surfactant composition. Oxygenation, lung mechanics, surfactant function and composition were significantly improved in the surfactant-treated group compared to the ventilated and VILI-control groups. We conclude that exogenous surfactant can be used to treat VILI.

Introduction

It is known that modes of mechanical ventilation which allow alveolar end-expiratory collapse and/or end-inspiratory alveolar overstretching lead to decreases in lung compliance¹⁻⁴ and gas exchange⁵, and result in atelectasis, pulmonary edema, pneumonitis and fibrosis^{6, 7}. Development of intra-alveolar edema in healthy rats subjected to intermittent positive pressure ventilation at high inflation pressures, without positive end-expiratory pressure (PEEP), was first demonstrated by Webb and Tierney and was later confirmed by Dreyfuss and colleagues who suggested that high inspiratory lung volumes induce endothelial and epithelial overstretching leading to microvascular injury^{8,9}. However, it is increasingly realized that impairment of the surfactant system plays a key role in the mechanism of ventilation-induced lung injury (VILI) in the above-mentioned model^{5, 10-12}; further on it has been shown that surfactant function is impaired by pulmonary edema constituents¹³⁻¹⁵. Loss of surfactant function will increase the surface tension at the air-liquid interphase of the alveolar walls^{1, 3}, which will lead, amongst others, to alveolar collapse and to an increased suction force on the pulmonary interstitium resulting also in alveolar edema^{5, 8-12}. Continuous re-expansion and collapse during the ventilatory cycles causes epithelial and endothelial damage mainly due to shear forces^{5, 10}. In addition, we have shown that exogenous surfactant administration preceding mechanical ventilation with high peak inspiratory lung volumes without PEEP, could partially prevent VILI which is characterized by e.g. impaired gas exchange and lung mechanics¹¹. In this study we wanted to investigate whether exogenous surfactant is able to restore gas exchange and lung mechanics in VILI.

Material and methods

Animal Preparation

This study was approved by the local Animal Committee at the Erasmus University Rotterdam, and the care and handling of the animals conformed with European Community guidelines (86/609/EC). The study was performed in 24 adult male Sprague-Dawley rats (body weight 280-350 g). Anesthesia was induced with 2% enflurane and 65% nitrous oxide in oxygen, a polyethylene catheter was inserted into a carotid artery for drawing arterial blood samples and continuous monitoring of arterial blood pressure. Immediately after, in a separate group of 6 animals a blood gas sample was taken and arterial blood pressure was measured, then the

animals were killed, the thorax was opened, and a static pressure-volume curve (P-V curve) was recorded and a bronchoalveolar lavage (BAL) was performed. These animals served as a non-VILI, non-ventilated control group (Healthy). In the remaining animals, before tracheostomy, the animals received 30 mg/kg pentobarbital sodium, i.p. (Nembutal[®], Algin BV, Maassluis, the Netherlands). After tracheostomy, muscle relaxation was induced by pancuronium bromide 0.6 mg/kg, i.m. (Pavulon[®], Organon Teknika, Boxtel, the Netherlands) immediately followed by connection to a ventilator and a pressure transducer for continuous monitoring of arterial blood pressure. The animals were mechanically ventilated with a Servo Ventilator 300 (Siemens-Elema, Solna, Sweden) in a pressure constant time-cycled mode, at an inspired oxygen concentration (FiO₂) of 1.0, frequency of 30 breaths per minute (bpm), peak inspiratory pressure (PIP) of 12 cm H₂O, positive end-expiratory pressure (PEEP) of 2 cm H₂O, and inspiratory/expiratory (I/E) ratio of 1:2. Anesthesia was maintained with pentobarbital sodium 30 mg/kg/h, i.p.; muscle relaxation was maintained with pancuronium bromide 0.6 mg/kg/h, i.m. Body temperature was kept within normal range by means of a heating pad.

Experimental Design

In order to produce VILI, PIP was increased to 45 cm H₂O and PEEP was decreased to zero for 20 min, other settings were not changed. Thereafter, PIP was decreased to 26 cm H₂O and PEEP was increased to 6 cm H₂O for 5 min, in order to increase arterial CO₂ tension. These ventilator settings were chosen based on a pilot study (unpublished data) in which we observed that when animals were ventilated at 45/0 cm H₂O (PIP/PEEP, respectively) for 20 minutes and then ventilated at 30/10 cm H₂O, the animals died from severe hypocapnia. Then, the animals were disconnected from the ventilator and the lungs were emptied of edema fluid and a randomization was performed.

Experimental Groups

The animals were randomized to one of three groups (n=6). The first group (Surfactant) received a bolus of exogenous surfactant (100 mg/kg) intratracheally. The surfactant used was isolated from minced pig lungs, that were processed as previously described [16](#). The surfactant suspension, at a concentration of 40 mg/ml, was administered as a bolus followed by a bolus of air 28 ml/kg, directly into the endotracheal tube via a syringe, and was immediately followed by re-connection to the ventilator. Mechanical ventilation was continued at a PIP of 30 cm H₂O, PEEP of 10 cm

H₂O, I/E ratio of 1:2, FiO₂ 1.0, and respiratory rate of 40 bpm for two hours. These ventilator settings, were chosen based on results of a preliminary study which showed that applied ventilation pressures of 26/6 cm H₂O (PIP, PEEP, respectively) and 28/8 cm H₂O were too low to keep animals alive for an observation period of 2 hours. The second group (Ventilated) did not receive exogenous surfactant but received a sham bolus of air 28 ml/kg intratracheally and was mechanically ventilated at the same settings as the Surfactant group. The third group of animals (VILI-control) were killed after the 5 minute ventilation period of 26/6 with an overdose of pentobarbital and were used as a non-treated, non-ventilated control group.

Gas Exchange and Hemodynamics

Arterial blood gas samples were taken in all groups before, after VILI, and at 5 min after the 26/6 period, and in the Surfactant and Ventilated control groups at 5 min after the 30/10 period, and every 30 min for 2 h. The samples were analyzed for arterial oxygen tension (PaO₂) and arterial carbon dioxide tension (PaCO₂) by conventional methods (ABL 505, Radiometer, Copenhagen, Denmark). At the same time points, arterial pressure was recorded. Hemodynamic support was provided by infusion of 1 ml of saline 0.9% (to a maximum of 2 ml per hour) when mean arterial pressure (MAP) decreased below 60 mmHg.

Pressure-Volume Curves

At 120 min after exogenous surfactant therapy all animals were killed with an overdose of pentobarbital sodium injected through the penile vein. Then static P-V curves were recorded. After the thorax and diaphragm were opened, the tracheostomy catheter was connected to a pressure transducer (Validyne model DP 45-32, Validyne Engineering Co., Northridge, CA, USA) with a syringe attached to it, and pressures were recorded on a polygraph (Grass model 7B, Grass Instrument Co., Quincy, MA., USA). Using a syringe filled with nitrogen (N₂) the lungs were first inflated (within 10 sec) to an airway pressure of 35 cm H₂O, which was maintained for 5 sec, followed by deflation to an airway pressure of 0 cm H₂O. Then the lungs were re-inflated in steps of 0.5 ml until an airway pressure of 35 cm H₂O was reached. Each inflation step took 1-2 sec followed by a 5-sec pause to allow pressure equilibration. After this, in the same way, the lungs were then deflated until an airway pressure of 0 cm H₂O was reached. The volume of N₂ left in the syringe was recorded. Maximal compliance (C_{max}) was calculated from the steepest part of

the deflation limb ¹⁶. Total lung capacity (TLC_{35}) was defined as lung volume at inflation with a distending pressure of 35 cm H₂O ¹⁷.

Gruenwald Index

The Gruenwald index which characterizes the surfactant system *in situ* ¹⁸, was calculated from the P-V curve, defined as $(2V_5+V_{10})/2V_{max}$, where V_5 , V_{10} and V_{max} are the lung volumes at transpulmonary pressures of 5, 10 and 35 cm H₂O from the deflation limb, respectively.

Functional Residual Capacity (FRC)

After P-V recordings, the lungs were removed *en bloc* and weighed, and lung volume at an airway pressure of 5 cm H₂O (V_5) was determined by fluid displacement. A positive pressure of 5 cm H₂O was chosen to compensate for the loss of transpulmonary pressure in the open chest ¹⁹. The total lung volume at this distending pressure was considered close to FRC.

Bronchoalveolar lavage

After the FRC measurement a BAL (30 ml/kg) was performed five times with saline-CaCl₂ 1.5 mmol/litre (crude lavage). Thereafter, cell debris were removed from BAL by centrifugation at 400 G for 10 min. The active surfactant component in the BAL fluid was separated from the non-active surfactant component by differential centrifugation, followed by subsequent phosphorus analysis, and the ratio of non-active to active (small to large aggregate) surfactant was calculated ²⁰. Finally, the protein concentration of the BAL fluid was determined using the Bradford method (Bio-Rad protein-assay, Munich, Germany) ²¹.

Minimal Surface Tension

Minimal surface tension of the crude lavage was determined by means of a modified Wilhelmy balance (E. Biegler GmbH, Mauerbach, Austria). In this method, a tight-fitting teflon barrier reduces the surface area of a teflon trough from 100-20% at a cycle speed of 0.33/min. Saline is used as subphase and is kept at 37°C. The force on a platinum slide (1x1 cm), dipped into the subphase, is measured by a force transducer and expressed as surface tension. Further, maximal surface tension is measured at 100% surface area and minimal surface tension at 80% surface compression and expressed as milli Newton/meter (mN/m). Surface tension characteristics of a BAL sample are measured after application on the surface of the saline-filled trough. In this

study 300 μ l of BAL fluid was applied to the surface of the trough; minimal surface tension was measured after 3 cycles ²².

Statistical data analysis

Statistical analysis was performed using the Instat 2.0 biostatistics package (GraphPad software, San Diego, CA, USA). Intragroup comparisons were analyzed with repeated measures ANOVA. Intergroup comparisons for protein concentration in the supernatant of BAL, total phosphorous of small aggregates, total phosphorous of large aggregates, non-active/active total phosphorous ratio, minimal surface tension of the crude lavage, C_{max} , TLC_{35} , Gruenwald index and V_5 were analysed by means of an ANOVA. If a $p < 0.05$ was found, a post-hoc test was performed (Tukey-Kramer). A t-test analysis was performed for intergroup comparisons during the 2-h study period, for PaO_2 , PCO_2 and MAP in the Surfactant and Ventilated control groups. Statistical significance was accepted at p -values < 0.05 . All data are expressed as mean \pm standard deviation.

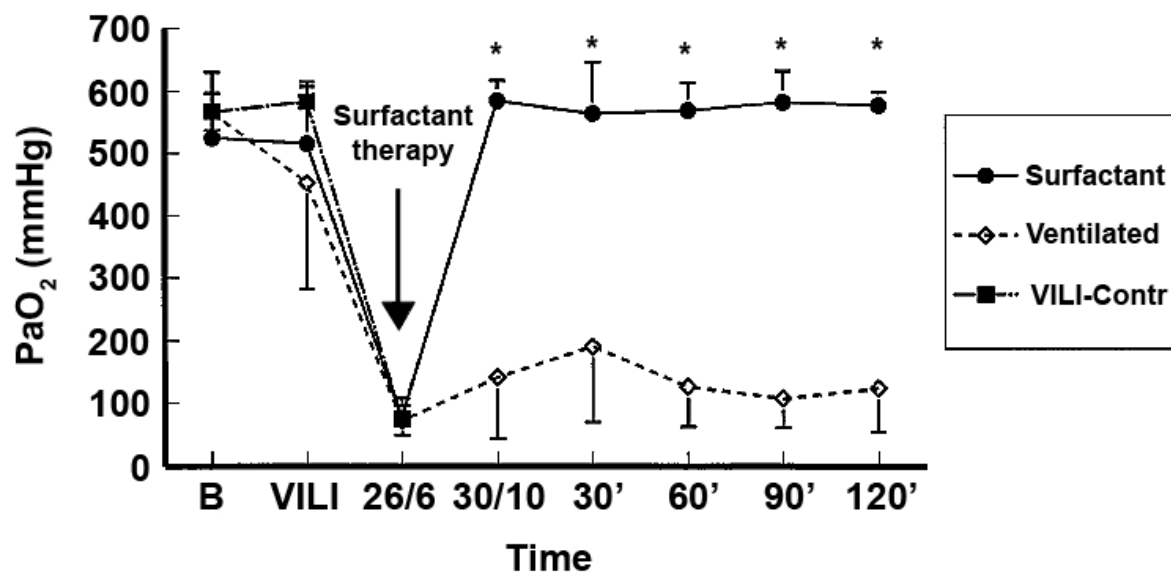


Figure 1. Arterial oxygen tension (mean \pm standard deviation) during the whole study period. B= basal, VILI= ventilation with Peak inspiratory pressure (PIP) of 45 cm H₂O without PEEP after 20 min, 26/6= after 5 min at PIP 26 cm H₂O, 6 cm H₂O PEEP, 30/10= 5 min PIP 30 cm H₂O, 10 cm H₂O PEEP. * indicates significant difference between the Surfactant group and the Ventilated control group.

Results

Figure 1 shows the PaO_2 levels during the whole study period. After the ventilator settings were set at 26/6 cm H₂O for 5 minutes the PaO_2 decreased below 100 torr in all animals. The Surfactant group showed a significant increase in PaO_2 values to pre-VILI levels ($p < 0.001$), and

were maintained during the 2-h study period. In the Ventilated control group, mean PaO₂ values remained below 200 torr during the 2-h study period: the difference between the values in the Ventilated group and the Surfactant group was significant throughout the study ($p < 0.001$).

Table 1. Data on arterial carbon dioxide tension (PaCO₂) and mean arterial pressure (MAP) over time

	Time	Healthy	VILI-control	Ventilated	Surfactant
PaCO ₂ (torr)	Basal	39±5	38±8	38±6	41±6
	VILI		14±3*	21±11*	14±2*
	5' 26/6		33±10	36±8	34±6
	5' 30/10			39±5	47±9
	30'			38±7	40±7
	60'			43±8	41±5
	90'			46±10	40±3
	120'			49±13	39±6
MAP (torr)	Basal	140±10	151±8	134±12	157±23
	VILI		77±26*	83±29*	89±31*
	5' 26/6		74±32*	68±43*	76±28*
	5' 30/10			106±20*	122±23
	30'			107±20	104±16
	60'			73±23*†	101±14
	90'			86±23	89±22
	120'			88±28	119±10

healthy control group (Healthy), non-Treated, non-Ventilated control group (VILI-control), Ventilated control group (Ventilated), and treated with surfactant group (Surfactant). Values are mean ± standard deviation.

* vs Baseline $p < 0.05$, † vs Surfactant $p < 0.05$

Table 1 shows that the PaCO₂ and MAP levels were comparable in both ventilated groups during the whole study period.

Table 2 shows data from BAL fluid and lung mechanics. Protein concentration was significantly higher in the three VILI groups when compared with Healthy controls. Additionally, protein concentration was significantly lower in the Surfactant group than in the VILI-control group, but not significantly different from the Ventilated control group. The ratio of small to large aggregates in BAL fluid was significantly lower in the Surfactant group compared to the VILI-control and the Ventilated control groups, and not different when compared with the Healthy group. The minimal surface tension of the crude lavage fluid in the Surfactant group was significantly lower than in the VILI-control and the Ventilated control groups. In the Surfactant group the Gruenwald index, TLC₃₅, and C_{max} were comparable with healthy values, and significantly higher than in the VILI-control and Ventilated control groups. However, V₅ values were significantly lower in the Surfactant group than in the Healthy control group, but significantly higher than in the VILI-control and Ventilated control groups.

Figure 2 shows the deflation limbs from the P-V curves. The Surfactant group had TLC₃₅, and C_{max} values comparable with the healthy group, and significantly higher than both the VILI-control and the ventilated control groups.

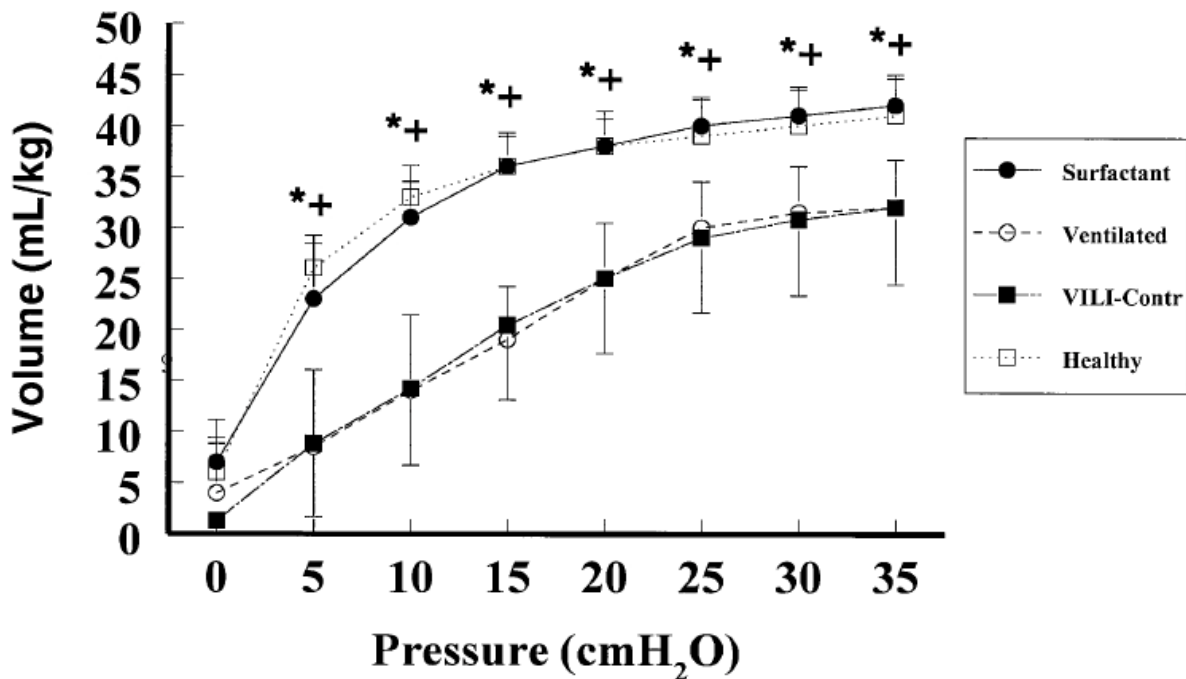


Figure 2. Deflation limbs from the pressure-volume curves, (mean ± standard deviation). * indicates significant difference between the Surfactant group and the two control groups, and + indicates significant difference between the Healthy group and the two control groups.

Table 2. Amount of recovered broncho-alveolar lavage (BAL) fluid, protein concentration, total phosphorus of small aggregates (SA) and total phosphorus of large aggregates (LA), non-active/active total phosphorus ratio (SA/LA ratio), minimal surface tension (min surf) of crude BAL fluid, Gruenwald Index, total lung volume at a transpulmonary pressure of 5 cm H₂O (V₅), lung volume above FRC at pressure 35 cm H₂O (TLC₃₅) and maximum compliance (C_{max}).

	Healthy	VILI-control	Ventilated	Surfactant
Recovery BAL fluid (%)	90±1	90±1	90±1	90±1
Protein concentration BAL (mg/ml)	0.3±0.1	0.9±0.03 ^{*†}	0.7±0.2 [†]	0.6±0.1 [†]
SA (mmol)	0.53±0.1 [*]	1.2±0.1 [*]	1.2±0.2 [*]	2.6±0.6
LA (mmol)	1.8±0.2 [*]	1.0±0.2 [*]	1.3±0.5 [*]	11±2
SA/LA ratio	0.39±0.05	1.3±0.22 ^{*†}	1.0±0.33 ^{*†}	0.22±0.08
Min surf (mN/m)	22.8±2.5 [*]	32.2±2.6 ^{*†}	29.5±1.1 ^{*†}	17.3±2.2
Gruenwald Index	1.0±0.01	0.20±0.08 ^{*†}	0.37±0.2 ^{*†}	0.96±0.06
V₅ (ml/kg)	24.3±5.6	3.5±0.5 ^{*†}	5.5±0.5 ^{*†}	13.0±1.0 [†]
TLC₃₅ (ml/kg)	41±3.6	32±8 ^{*†}	32±5 ^{*†}	42±3
C_{max} (ml/kg)	4±0.5	1.8±0.7 ^{*†}	1.6±0.3 ^{*†}	3.2±0.7

Values are mean ± standard deviation, * p< 0.05 vs Surfactant, † p< 0.05 vs Healthy

Discussion

This study shows that exogenous surfactant given to rats suffering from VILI restored the gas exchange at the used ventilator settings to basal values, and improved lung mechanics.

In the present study, 20 minutes of ventilation with high peak-inspiratory pressures without PEEP resulted in pulmonary edema and hypoxemia, and in impairment of the surfactant system. The latter is characterized by a decrease in pulmonary compliance, V₅, and Gruenwald Index. The exact mechanism by which the lung damage is produced by artificial ventilation is not yet entirely clear, but the role of surfactant changes is increasingly realised [1-5, 11, 12, 23, 24](#). Two primary mechanisms of surfactant inactivation by mechanical ventilation have been described. In the first mechanism mechanical ventilation enhances surfactant release from the pneumocytes type II into the alveolus [1-4](#). This material is subsequently lost into the small airways as a result of

compression of the surfactant film when the surface of the alveolus becomes smaller than the surface occupied by the surfactant molecules, so that surface active material moves into the airways [1](#), [4](#), [24](#). The second mechanism describing the surfactant changes associated with mechanical ventilation is based on the observation that the alveolar surface area changes associated with mechanical ventilation result in the conversion of large surface-active surfactant aggregates into small nonsurface-active surfactant aggregates [5](#), [25](#), [26](#). These two mechanisms will lead to alveolar collapse and protein infiltration [5](#), [8-12](#) in which the latter leads to further inactivation of surfactant [13-15](#). These mechanisms produce self-perpetuating changes which require higher ventilator pressures which may finally be responsible for more parenchymal damage [5](#), [8-12](#).

In the current study we used an exogenous surfactant to replace the surfactant lost and/or inactivated during VILI, trying to re-establish the physiological surface tension at the air-liquid interface. The exogenous surfactant used contains 1-2% of the surfactant proteins B and C which are a pre-requisite for a rapid adsorption at the air-liquid interface. In the Surfactant group a significant increase in arterial oxygen tension levels, comparable with basal values, was seen within 5 minutes and was sustained during the 2-h study period. At the end of the study period TLC_{35} , C_{max} , and the Gruenwald index, were significantly higher in the Surfactant group compared with the Ventilated and VILI-control groups, and not significantly different from the healthy control group. Additionally, in the Surfactant group a low minimal surface tension of the BAL fluid was observed. It is known that one of the most important functions of the pulmonary surfactant system is the mechanical stabilisation of the lung alveoli during end-expiration. This is achieved by decreasing the surface tension in parallel with the decrease in alveolar radius [27](#). Conversely, a high surface tension will promote alveolar collapse during deflation of the lung [1-4](#), [8-10](#). Based on our results, we assume that the alveolar surface tension was restored by exogenous surfactant in the Surfactant group, providing, together with PEEP, open alveoli resulting in almost normal arterial oxygen tension. In contrast, the Ventilated control group showed an impaired gas exchange and decreased alveolar stability, probably caused by the demonstrated high surface tension in the BAL fluid of this group.

Another important function of pulmonary surfactant is the stabilization of fluid balance in the lung and preventing pulmonary edema [27-29](#). Therefore, loss of surfactant function will lead to alveolar edema which dilutes and inactivates the pulmonary surfactant. The protein level from the crude BAL fluid was significantly higher in all exposed to VILI groups compared with the

Healthy group. Therefore the application of exogenous surfactant had no additional effect on the resolution of this edema during the 2-h study period. More studies have to be performed to determine if there are any changes in lung water, microvascular permeability, and histological parameters of edema when exogenous surfactant is used after VILI.

The model of VILI used in this study might resemble a clinical situation, especially when high inspiratory lung volumes are applied. It is known that mechanical ventilation may damage the lung in the presence or absence of pre-existing lung disease and produces a similar pattern of injury as that observed during ARDS ²³; mechanical ventilation can induce lung parenchymal damage especially in the surfactant deficient parts of the ARDS lungs and may further induce surfactant changes in those parts of the ARDS lung which still have an adequately functioning surfactant system ¹². The possible clinical relevance of our study is that exogenous surfactant can be used not only to prevent VILI, but also as a treatment after VILI, restoring the surfactant function in those alveolar units already damaged and preventing damage of the intact alveolar units.

In conclusion, our results show that exogenous surfactant can be used as a treatment for VILI, restoring lung function and lung mechanics.

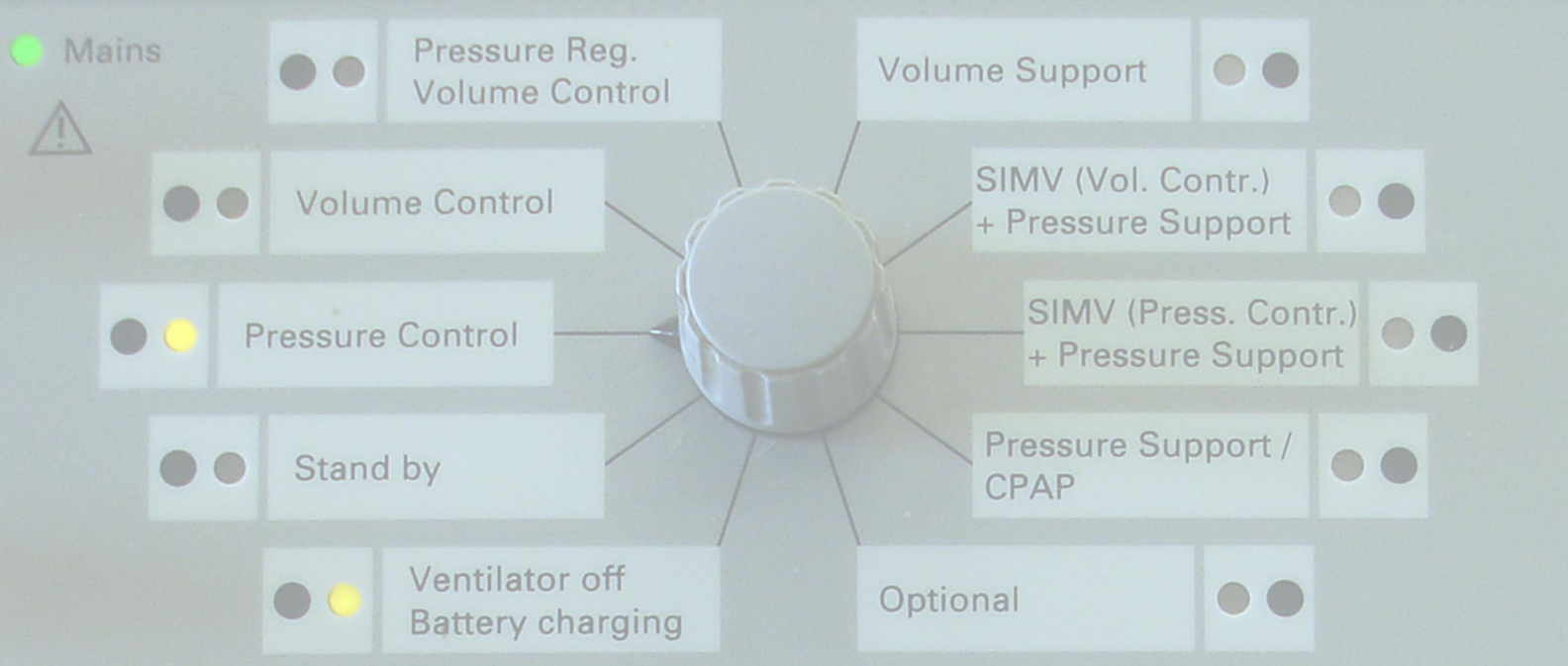
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SURFACT





Resp. pattern

Measured freq.
b/min
50

Set freq.
b/min
50

Insp. period
s
0.40

Insp. flow
l/s

Chapter 12

Summary and conclusions

Summary

Chapter 1 provides a review of the acute respiratory distress syndrome (ARDS) focusing on the roles of the surfactant system and the effects of mechanical ventilation on the development of ARDS. This chapter also summarizes the pathophysiology of ventilator-induced lung injury (VILI) in which surfactant changes play a key role. It is concluded that the need to ventilate patients with acute respiratory failure may initiate the following vicious circle: Surfactant inhibition due to mechanical ventilation (VILI) will lead to increased surface tension with increased protein leakage and decreased lung function. Consequently, respiratory failure will get worse requiring intensified ventilatory support, therefore increasing the likelihood for further VILI completing the vicious circle. Therefore, ventilation strategies should comply with one rational concept which prevents damage due to mechanical ventilation itself. “Open up the whole lung and keep it open” with the least possible influence on the cardio-circulatory system. Recent animal and clinical studies suggest that such a protective lung strategy may prevent the release of inflammatory mediators from the lung and the transfer of bacteria and bacterial endotoxins into the bloodstream, and may have an important beneficial effect on mortality rates of ARDS. **Chapter 2** focuses on surfactant replacement therapy which seems a promising approach for the treatment of acute respiratory failure in ARDS and ARDS-like syndromes. An overview is presented of experimental models of surfactant dysfunction as well as the effects of exogenous surfactant in these models. Finally, unanswered questions concerning surfactant treatment are discussed and a summary of surfactant replacement therapy in clinical trials is presented.

Chapter 3 explores the diagnostic properties of liver-type fatty acid binding protein (L-FABP) in serum to indicate early lung tissue damage. After induction of experimental lung injury by whole lung lavage, rats were ventilated for 4 hours having either received exogenous surfactant or no treatment, while one group served as non-lavaged control animals. After 2 hours of ventilation we could demonstrate that L-FABP serum concentrations were highest in the non-treated lavage animals, emphasizing an early correlation between the degree of lung tissue damage and the appearance of L-FABP in serum. The role of alveolar macrophages and type II pneumocytes are discussed as possible sources of the measured L-FABP.

The study presented in **Chapter 4** was designed to identify the cell types and specific signaling mechanisms that are activated by ventilation with increased pressure/volume (overventilation). *Ex vivo* and *in vivo* overventilation of lung tissue caused nuclear translocation of nuclear factor- κ B (NF- κ B) and enhanced expression of interleukin-6 (IL-6) mRNA in alveolar macrophages (AM) and alveolar epithelial type II (ATII) cells. The phosphoinositide 3-OH kinase (PI3K) inhibitor Ly294002 prevented nuclear translocation of NF- κ B and the subsequent release of IL-6 and macrophage inflammatory protein-2 α (MIP-2 α) in overventilated, but not in lipopolysaccharide (LPS) induced endotoxic lungs. These findings show that AM and ATII cells contribute to the ventilation-induced release of pro-inflammatory mediators and that selective inhibition of this process is possible without inhibiting the activation of NF- κ B by endotoxin.

Chapter 5 investigates the influence of surfactant and “Open Lung Ventilation” on bacterial growth in lungs and on the degree of bacteremia in experimental group B streptococcal (GBS) pneumonia. Newborn piglets were lung lavaged after which some of the animals received exogenous surfactant treatment. Thereafter, experimental pneumonia was induced by intratracheal instillation of a GBS solution. The animals were ventilated with either conventional or “open lung” ventilatory settings for 5 hours. This study showed that strategies preventing atelectasis formation by either surfactant therapy and/or “open lung” ventilation reduced bacterial growth in the lung as well as bacterial translocation from the lung into the systemic circulation compared to strategies that allowed atelectasis formation.

The question whether the prevention of atelectasis formation is the only factor that determines bacterial translocation from the lung into the bloodstream is investigated in **Chapter 6**. Similar to the study in Chapter 5, newborn piglets received a solution of GBS after whole lung lavage. The animals were then ventilated for 5 hours in three different modes, either a conventional mode or “open lung” mode or high positive endexpiratory pressure (PEEP) mode (i.e. using higher pressures than required for optimal gas exchange). We found that both conventional (atelectasis prone) and high PEEP (little or no atelectasis) animals had comparable higher bacterial translocation rates compared to optimal “open lung” ventilation, emphasizing that the degree of atelectasis in lungs (as stated in Chapter 5) may not be the only factor for increased bacterial translocation, but also increased constant stretch on the alveolar-capillary membrane due to high end-inspiratory and end-expiratory lung volumes generated by high PEEP ventilation.

The high incidence and prevalence of ventilator-associated pneumonia on today's intensive care units poses a great threat to ventilated patients, increasing their probability to develop septicemia due to bacterial translocation. **Chapter 7** and **Chapter 8** deal with different treatment approaches for either pneumonia and/or pneumonia-induced bacteremia. In **Chapter 7**, rats with respiratory failure received a high or a low dose of intravenous immunoglobulin M-enriched immunoglobulins before intratracheal instillation of a *Klebsiella pneumoniae* solution and ventilation for 3 hours. It was demonstrated that the preventive immunoglobulin therapy was able to neutralize translocated *K. pneumoniae* from the lung into the bloodstream in a dose-dependent way.

In **Chapter 8**, the intratracheal antibiotic drug delivery properties of surfactant is compared to systemic antimicrobial treatment in rats with a *Klebsiella pneumoniae* infection. This study showed that surfactant used as intratracheal carrier for a given tobramycin dose is superior in clearing bacteria from lungs compared to the same dose given intravenously. However, intratracheal surfactant therapy alone without tobramycin increased bacterial growth in lungs and mortality, questioning the rationale to use exogenous surfactant as treatment for pneumonia.

The loss of surfactant function due to lower surface tension at the air/liquid interface at the alveolar level is a common etiological finding in acute lung injury and a component of the vicious circle mentioned in Chapter 1. Chapters 9-11 explore different therapeutical interventions that target this pathologically increased surface tension.

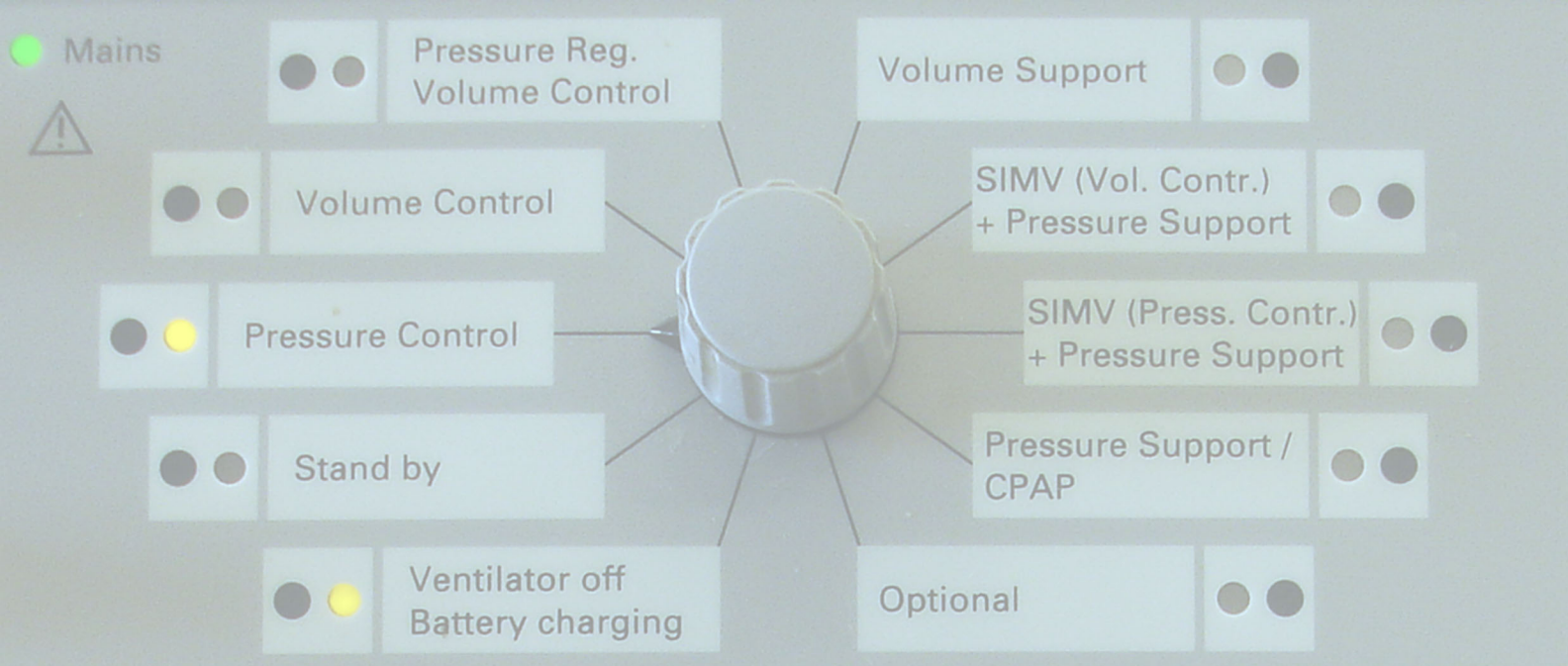
Chapter 9 investigated the effect of partial liquid ventilation (PLV) on lung function in VILI-induced lung injury. During PLV lungs are partially filled up with perfluorocarbons (PFCs) with superimposed gas ventilation. The surfactant-like surface tension lowering activity and the capability to dissolve high amounts of oxygen and carbon dioxide, allow gas exchange over the alveolo-capillary barrier to continue in PFC-filled lungs. In this study, in rats with VILI 2 hours of PLV with different PFC dosages improved gas exchange and pulmonary compliance in a dose-dependent way, again emphasizing the importance of low surface tensions at the alveoli air/liquid interface for normal gas exchange to take place. **Chapter 10** explores the role of PFCs low surface tension property on opening pressures needed to recruit atelectatic lung areas. In this study, pigs with acute lung injury received either a small dose of intratracheal PFC or no treatment followed by a recruitment maneuver. It was demonstrated that the instillation of a small amount of PFC before performing an open lung maneuver significantly reduced the pressures needed to recruit atelectatic lung regions.

The rationale to treat surfactant dysfunction - the common cause of acute lung injury - with exogenous surfactant is tested in **Chapter 11**. Rats with VILI received either an intratracheal bolus of exogenous surfactant or no treatment, after which the animals were ventilated for 2 hours with the same ventilation settings. Arterial PO₂ improved immediately to healthy baseline values in surfactant-treated animals while the PaO₂ remained at low levels in non-treated animals. Surfactant treatment also resulted in improved lung mechanics, surfactant function and composition leading to the overall conclusion that exogenous surfactant can be used to treat VILI.

Conclusions

In conclusion, the studies in this thesis provide insights into different aspects (pathophysiology, diagnosis and therapy) of acute lung injury. We could demonstrate an association between serum L-FABP concentrations and the degree of lung cell damage, suggesting a possible diagnostic value of L-FABP to indicate early lung cell damage. While alveolar macrophages and type II pneumocytes are discussed to be the source of the L-FABP, these two cell types were clearly identified to contribute to the ventilation-induced release of pro-inflammatory mediators. Furthermore, the application of a PI3K inhibitor selectively blocked the overinflation induced release of these mediators but had no effect on LPS-induced endotoxic lungs, suggesting that it may be possible to block biotrauma without affecting innate immunity. We showed that mechanical ventilation may be the cause of bacterial translocation from the lung into the bloodstream in acute lung injury with pneumonia, emphasizing that this translocation can be minimized by using an optimal ventilation strategy (open lung concept). Alternatively, the intravenous application of IgM-enriched immunoglobulins proved to be a valuable therapy to neutralize translocated bacteria. Further, treating the underlying pneumonia was most efficient when the given antibiotic (tobramycin) dose was intratracheally instilled using surfactant as carrier as compared to systemic antimicrobial therapy. Finally, we could show that both PLV and exogenous surfactant can be used to treat VILI, although surfactant was more effective due to the fact that it directly improves the underlying cause of VILI, namely the surfactant dysfunction itself.





Resp. pattern

Measured freq. b/min

50

Set freq. b/min

50

Insp. period s

0.40

Insp. flow l/s

1.00

Chapter 13

Samenvatting

In **Hoofdstuk 1** wordt een overzicht gegeven van het “Acute Respiratory Distress Syndrome” (ARDS) waarin met name de rol van het surfactantsysteem en het effect van mechanische beademing op de ontwikkeling van ARDS wordt beschreven. Dit hoofdstuk geeft ook een samenvatting van de pathofysiologie van door beademing geïnduceerde longschade (VILI). Hierbij spelen veranderingen van surfactant een grote rol. Er is bewezen dat patiënten met acuut respiratoirfalen, waarbij beademing noodzakelijk is, in een volgende vicieuze cirkel terechtkomen. Inhibitie van surfactant door mechanische beademing (VILI) zal leiden tot een toename van de oppervlaktespanning met een toename van eiwitlekage en een vermindering van longfunctie tot gevolg. Daardoor zal het respiratoirfalen verder toenemen, waardoor intensievere beademingsondersteuning nodig is. Als gevolg daarvan zal naar alle waarschijnlijkheid, door een verdere toename van VILI de vicieuze cirkel compleet zijn. Daarom zouden beademingsstrategieën moeten voldoen aan een rationeel concept waarbij de preventie van schade, ontstaan door mechanische beademing, voorop staat. “Open de gehele long en houdt hem open” met de minst mogelijke invloed op het cardiocirculaire systeem. Recente klinische studies en studies met diermodellen suggereren dat zo’n longbeschermingsstrategie mogelijk het vrijkomen van inflammatoire mediators vanuit de long voorkomt, zodat er geen overdracht van bacteriën en bacteriële endotoxines naar het bloed plaatsvindt. Dit heeft waarschijnlijk een gunstig effect op het sterftcijfer van ARDS.

In **Hoofdstuk 2** wordt de nadruk gelegd op de surfactantvervangings therapie, een mogelijk veelbelovende behandeling bij acuut respiratoirfalen in ARDS en ARDS-achtige ziektebeelden. Ook worden in dit hoofdstuk experimentele modellen besproken waarbij het functieverlies van surfactant en de effecten van exogeen surfactant op deze modellen een rol spelen. Tenslotte worden onbeantwoorde vragen besproken betreffende de surfactantbehandeling. Tevens wordt er een samenvatting van de surfactantvervangings therapie in klinische onderzoeken gepresenteerd.

In **Hoofdstuk 3** worden de diagnostische eigenschappen van lever-type vetzuur bindingseiwit (L-FABP) in het serum onderzocht. Als L-FABP voorkomt in het serum wijst dit mogelijk op een vroegtijdige beschadiging van het longweefsel. Door het laveren van de longen werd bij de ratten een experimentele longschade geïnduceerd. Daarna werden de dieren vier uur lang beademd, waarbij een groep met exogeen surfactant en een groep zonder behandeld werden. De controlegroep bestond uit ratten waarbij de longen niet

gelaveerd waren. Na twee uur beademen werden de hoogste concentraties van het L-FABP in het serum van de onbehandelde, niet gelaveerde dieren gevonden. Hiermee wordt een vroegtijdige correlatie tussen de mate van schade in het longweefsel en het verschijnen van L-FABP in het serum aangetoond. De rol van alveolaire macrofagen en type II pneumocyten wordt gezien als mogelijke bron van de gemeten concentraties L-FABP in het serum.

De studie die gepresenteerd wordt in **Hoofdstuk 4** is ontworpen om de celtypen en specifieke signaleringsmechanismen, die worden geactiveerd door beademing met hoge drukken en grote volumes te identificeren. Er is dan sprake van overbeademing. *Ex vivo* en *in vivo* overbeademing van longweefsel veroorzaakt nucleaire translocatie van nucleair factor- κ B (NF- κ B) en versterkt de expressie van interleukine-6 (IL-6) mRNA in alveolaire macrofagen (AM) en alveolaire epitheel type II (ATII) cellen. De phosphoinositide 3-OH kinase (PI3K) inhibitor Ly294002 verhindert nucleaire translocatie van NF- κ B en het daarop volgend vrijkomen van IL-6 en het macrofage inflammatoire eiwit-2 α (MIP-2 α). Dit wordt gezien bij overbeademing, maar niet bij lipopolysaccharide (LPS) geïnduceerde endotoxische longen. Deze bevindingen laten zien dat AM en ATII cellen bijdragen aan het vrijkomen van pro-inflammatoire mediators geïnduceerd door beademing. En dat selectieve inhibitie van dit proces mogelijk is zonder inhibitie van NF- κ B door endotoxine.

In **Hoofdstuk 5** wordt de invloed van surfactant en de “open long” beademing op bacteriegroei in de long en de mate van bacteriëmie besproken in groep B Streptococcus (GBS) pneumoniae. Bij pasgeboren biggetjes werden de longen gelaveerd. Enkele dieren werden behandeld met exogeen surfactant. Daarna werd experimentele pneumonie geïnduceerd door intratracheale installatie van een GBS-oplossing. De dieren werden vijf uur beademd met een conventionele manier van beademen of met de “open long” beademingsmethode. Deze studie laat zien dat bacteriegroei in de long en ook de translocatie van bacteriën vanuit de long naar de bloedcirculatie gereduceerd wordt door beademingsstrategieën die formaties van atelectase voorkomen, zoals surfactanttherapie en/of “open long” beademing. Dit in vergelijking met strategieën waarbij wel formatie van atelectase wordt toegestaan.

De vraag of de preventie van formaties van atelectase de enige beslissende factor is in het beëindigen van translocatie van bacteriën vanuit de long naar de bloedcirculatie, is onderzocht in **Hoofdstuk 6**. Gelijk aan de studie in hoofdstuk 5, kregen pasgeboren biggetjes na lavage van de gehele long een GBS-oplossing toegediend. Daarna werden de dieren vijf uur beademd met drie verschillende methodes: met een conventionele beademingsmethode,

een “open long” beademingsmethode en met een hoge positieve eindexpiratoire drukbeademing (PEEP). De laatste is een beademingsmethode waarbij hogere drukken worden gehanteerd dan nodig zijn voor optimale gasuitwisseling. De uitkomst van deze studie was, dat zowel bij de conventionele beademingsmethode (atelectase gevoelig) als bij de hoge PEEP beademingsmethode (weinig tot geen atelectase) de dieren een vergelijkbare hoge mate van translocatie van bacteriën vertoonden. Dit in vergelijking met de optimale “open long” beademing. Hierbij moet benadrukt worden dat de mate van atelectase in de longen (zoals eerder beschreven in hoofdstuk 5) dus niet de enige factor is voor een toename van translocatie van bacteriën, maar dat een constante oprekking van het alveolaire capillaire membraan ook een rol speelt. Dit is ontstaan door beademing met hoge PEEP, waarbij hoge eindexpiratoire en hoge eindexpiratoire longvolumes gegenereerd worden.

De hoge voorvallen en hoge algemeen voorkomende gevallen van pneumonie, die geassocieerd worden met beademing, vormen tegenwoordig een grote bedreiging voor patiënten die beademd moeten worden op de intensive care unit. Naar alle waarschijnlijkheid is de kans groot dat deze groep patiënten een septikemie ontwikkelt door translocatie van bacteriën.

In **Hoofdstuk 7** en **Hoofdstuk 8** worden verschillende behandelingsmethodes besproken voor zowel pneumonie als bacteriëmie veroorzaakt door een pneumonie. In **Hoofdstuk 7** werd intraveneus een hoge dosis en een lage dosis van immunoglobulinen verrijkt met immunoglobuline-M (IgM) aan ratten toegediend met respiratoirfalen. Daarna vond er een intratracheale installatie met een *Klebsiella pneumoniae* oplossing plaats en werden de dieren drie uur lang beademd. De resultaten uit dit onderzoek bewijzen dat het mogelijk is om met een immunoglobulinetherapie de translocatie van *Klebsiella pneumoniae* vanuit de long naar de bloedcirculatie op een dosisafhankelijke wijze te neutraliseren.

In **Hoofdstuk 8** wordt een eigenschap van de surfactantbehandeling, namelijk intratracheale antibioticumoverdracht, vergeleken met een systemische antimicrobische behandeling bij ratten met een *K. pneumoniae* infectie. Deze studie laat zien dat wanneer surfactant gebruikt wordt als intratracheale drager voor een toegediende dosering van tobramycine, de klaring van bacteriën vanuit de long beter is dan wanneer diezelfde dosis tobramycine intraveneus wordt toegediend. Als hoe dan ook de intratracheale surfactanttherapie zonder tobramycine een verhoging van de bacteriegroei in de longen en een verhoogde mortaliteit laat zien, resteert de vraag of er een gegronde reden bestaat om patiënten met een pneumonie te behandelen met exogeen surfactant.

Het functieverlies van surfactant, door een verlaging van de oppervlaktespanning op het lucht-vloeistofscheidingsvlak op alveolair niveau, is een bekende ethologische vinding bij acute longschade en daarnaast een component uit de vicieuze cirkel genoemd in hoofdstuk 1. In de hoofdstukken 9 tot en met 11 worden verschillende therapeutische interventies onderzocht die streven naar een pathologische verhoging van de oppervlaktespanning.

In **Hoofdstuk 9** wordt het effect van partiële vloeistofbeademing (PLV) op de longfunctie onderzocht bij door VILI geïnduceerde longschade. Tijdens de PLV worden de longen gedeeltelijk gevuld met perfluorcarbonen (PFC's) en daarnaast nog beademd met gas. De surfactantachtige oppervlaktespanningverlagende activiteit en het vermogen van PFC's om grote hoeveelheden zuurstof en koolstofdioxide op te lossen, zorgen ervoor dat gasuitwisseling wordt voortgezet in de alveolocapillaire barrière van de met PFC gevulde longen. In de studie die in dit hoofdstuk beschreven staat, werden ratten met VILI twee uur beademd met PLV. De verschillende PFC doseringen verbeterden de gasuitwisseling en de pulmonaire compliantie op een dosisafhankelijke wijze. Hierbij wordt weer de nadruk gelegd op het belang van een verlaagde oppervlaktespanning op het lucht-vloeistofscheidingsvlak van de alveoli zodat een normale gasuitwisseling plaats kan vinden.

In **Hoofdstuk 10** is onderzocht wat de rol is van een specifieke eigenschap van PFC's op de openingsdrukken, die nodig zijn om atelectase-achtige gebieden in de long te kunnen recrutereren. De eigenschap waar het hier om gaat, is de mogelijkheid van PFC's om de oppervlaktespanning te verlagen. In deze studie ontvingen varkens met acute longschade een lage dosis van intratracheale PFC of werden niet behandeld. Achtereenvolgens werd een recruitmentmanoeuvre uitgevoerd. Deze studie laat zien dat een installatie van een kleine hoeveelheid PFC, voordat er een longopeningsmanoeuvre wordt uitgevoerd, de drukken die nodig zijn om atelectase-achtige gebieden in de long te recrutereren significant verlagen.

Dat het verstandig is om bij het disfunctioneren van surfactant, wat het meest voorkomt bij acute longschade, tot behandeling met exogeen surfactant over te gaan is getest in **Hoofdstuk 11**. Ratten met VILI ontvingen ofwel een intratracheale bolus van exogeen surfactant of werden niet behandeld met surfactant. Daarna werden ze twee uur beademd met gelijke beademingsinstellingen. De groepen die behandeld waren met surfactant, lieten een verbetering zien van de arteriële PO_2 (gelijk aan gezonde baselinewaarden).

Bij de groep die niet behandeld was met surfactant, bleven de PaO_2 -waarden laag. De behandeling met surfactant leidt tot verbeterde longmechanismen, surfactantfunctie en surfactantcompositie. Hieruit kunnen we globaal concluderen dat exogeen surfactant gebruikt kan worden voor de behandeling van VILI.

Conclusies

Tot besluit kunnen we concluderen dat de studies beschreven in dit proefschrift een inzicht geven in de verschillende aspecten (pathofysiologie, diagnose en therapie) van acute longschade. We hebben laten zien dat er een correlatie bestaat tussen de L-FABP concentraties in het serum en de mate van longcelschade. Hiermee geven we in overweging dat de diagnostische waarde van L-FABP een mogelijke indicatie is voor vroegtijdige longcelschade. Terwijl het nog ter discussie staat of alveolaire macrofagen en type II pneumocyten de bron zijn van de L-FABP, hebben wij duidelijk aangetoond dat deze twee celtypen een bijdrage leveren aan het vrijkomen van door beademing veroorzaakte pro-inflammatoire mediators. Verder hebben we aangetoond dat het gebruik van een PI3K inhibitor selectief de mediators blokkeert die vrijkomen bij “overinflation”. Dit effect wordt niet gezien in door LPS geïnduceerde endotoxische longen. Hiermee wordt gesuggereerd dat het mogelijk is om biotrauma te blokkeren zonder de aangeboren immuniteit aan te tasten. Ook hebben we laten zien dat mechanische beademing mogelijk een oorzaak is van bacteriële translocatie vanuit de long naar de bloedsomloop bij acute longschade met pneumonie. Hierbij willen we benadrukken dat deze translocatie tot een minimum beperkt kan worden door gebruik te maken van een optimale beademingsstrategie, namelijk het open long concept. In het andere geval hebben we bewezen dat het intraveneus gebruik van immunoglobulinen verrijkt met IgM een waardevolle therapie kan zijn bij het neutraliseren van getransloceerde bacteriën. Verder hebben we laten zien dat het behandelen van een onderliggende pneumonie het meest efficiënt is als het antibioticum (tobramycine) intratracheaal gegeven wordt, waarbij het surfactant gebruikt wordt als drager. Dit in vergelijking met een systemische antimicrobische therapie. Tot slot hebben we aangetoond dat zowel PLV als het exogeen surfactant gebruikt kan worden om VILI te behandelen. Surfactant is echter effectiever vanwege het feit dat het direct de onderliggende oorzaak van VILI aanpakt, namelijk de surfactantdisfunctie zelf.



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The writing of the acknowledgements is supposed to be the most difficult part of the *Promotieboek*. This is at least the impression I got after having read quite a lot of them. Well, I can't tell yet because I am still at the very beginning. But I think the first difficulties I ran into started even before I had actually typed anything and it had something to do with the Dutch word for acknowledgements. Because *Dankwoord* sounds like a proper German word (but it's not) I started to use it as such which got me into trouble with my mother. Anyways, having thought about all the people who helped me in accomplishing this thesis I find it difficult to really weigh my thanks. Everybody who supported me was part of the chain called *promotion*, some links obviously stronger and thicker than others but still, without even the weakest link the chain would have been broken. Having said this, the following does not necessarily represent an order of importance but rather a grouping of content.

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world when necessary, within the blink of an eye - a truly remarkable and extraordinary property of yours. Good persons always prevail, so better prepare for a loooooong life.

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Curriculum vitae

Robert Lachmann was born on April 20th 1975 in (East)Berlin, Germany. He graduated from gymnasium receiving his “Abitur” in July 1994 (Herbartgymnasium, Oldenburg, Germany). In the same month he was drafted to serve as a paramedic in the German army. In 1995, he started medical school at the Free University of Berlin changing to the Humboldt University Berlin-Charité two years later. He graduated as a medical doctor in 2003. In the same year he commenced his work as full-time researcher and PhD candidate at the Department of Experimental Anesthesiology, Erasmus University Rotterdam. From November 2006 on he will most likely be traveling the planet before eventually starting clinical work.



The photos at the end of the articles are all somehow (locally and/or concerning the contents) related to the “afdeling Experimentele Anesthesiologie” on the 23rd floor of the Erasmus MC-Faculty in Rotterdam, The Netherlands, ...except the last one.