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Postpneumonectomy pulmonary edema and the nitric oxide pathway
An experimental study

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A dissertation for the degree Philosophiae Doctor in Medical Science

To my family…

Contents   **Pages**

1. ACKNOWLEDGEMENTS

2. ABSTRACT

Background
Postpneumonectomy pulmonary edema (PPE) developing independently of left ventricular dysfunction, fluid overload or infection is a dangerous type of acute lung injury (ALI). Despite efforts to unveil its etiology and pathogenesis, PPE remains an elusively understood condition with a high mortality.

Mechanical ventilation (MV) with high tidal volumes and low end-expiratory pressure may result in damage to the lungs, referred to as ventilator - induced lung injury (VILI). Investigations on isolated rabbit lungs have shown that the NO synthase (NOS) inhibitor L-NAME blunted the increase in lung microvascular permeability and lipid peroxidation after VILI. Our group has shown that methylene blue (MB), an inhibitor of constitutive and inducible NOS, modulates ovine endotoxin-induced lung injury. Correspondingly, investigators have noticed that 7-nitroindazole (NI), an inhibitor of neuronal NOS (nNOS), attenuates ovine lung injury models based on smoke inhalation alone, or in combination with live bacteria into the airways triggering pneumonia and sepsis. We speculated if pneumonectomy (PE) followed by excessive ventilation of the remaining lung in combination with zero end-expiratory pressure promotes VILI and that this subtype of ALI might be dampened by the inhibitors of NOS, MB and NI.

Methods
Anesthetized sheep underwent pneumonectomy followed by randomization to a protectively ventilated group with tidal volumes of 6 mL/kg, FiO_2 of 0.5 and positive end-expiratory pressure (PEEP) of 2-4 cm H_2O (PROTV group), or injurious ventilation of 12 mL/kg and zero end-expiratory pressure (INJV group) keeping $PaCO_2$ within normal limits (Paper I). The sheep studied in Papers II and III underwent the same protocol with the exception that one subgroup of injuriously ventilated animals was exposed to intravenous infusion of MB (INJV+MB group) from one hour after the onset of injurious ventilation (Paper II), or to infusion of the inhibitor of neuronal NOS, 7-nitroindazole (INJV+NI group) from two hours after the commencement of injurious ventilation (Paper III).

Results
Following pneumonectomy, the change of lung ventilation from a protective mode to an injurious mode with zero end-expiratory pressure (ZEEP), caused a significant rise in extravascular lung water (EVLW), as demonstrated in Paper I. We failed to demonstrate any effects of MB on the changes to injurious ventilation after PE (Paper II). Inhibition of nNOS with NI improved gas exchange, but did not reduce lung water extravasation due to excessive ventilation after PE (Paper III).

Conclusion
In conclusion, injurious ventilation with zero end-expiratory pressure caused a significant rise in EVLW and derangement of gas exchange that was uninfluenced by MB. In contrast, inhibition of nNOS improved gas exchange, most likely by reinforcement of hypoxic pulmonary vasoconstriction, but did not influence EVLW. However, our experiments do not indicate a major role in the pathogenesis of PPE for NO or its metabolites.

3. LIST OF PAPERS

Paper I
V.V. Kuzkov, E.V. Suborov, M.Y. Kirov, V.N. Kuklin, M. Sobhkhez, S. Johnsen, K. Waerhaug and L.J. Bjertnaes, 2007, **Extravascular lung water after pneumonectomy and one-lung ventilation in sheep**: *Crit Care Med, v. 35, no. 6, p. 1550–9.*

Paper II
E.V. Suborov, V.V. Kuzkov, M. Sobhkhez, M.Y. Kirov and L.J. Bjertnaes, 2010, **The effects of methylene blue on ovine post-pneumonectomy pulmonary oedema:** *Acta Anaesthesiol Scand, v. 54, no. 9, p. 1089-96.*

Paper III

E.V. Suborov, A.A. Smetkin, T.V. Kondratiev, A.Y. Valkov, V.V. Kuzkov, M.Y. Kirov and L.J. Bjertnaes, 2012, **Inhibitor of neuronal nitric oxide synthase improves gas exchange in ovine ventilator-induced lung injury after pneumonectomy:** *BMC Anesthesiology 12:10 doi:10.1186/1471-2253-12-10*

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4. ABBREVIATIONS

7-NI	7-nitroinidazole
ALI	Acute lung injury
ANP	Atrial natriuretic peptide
ARDS	Acute respiratory distress syndrome
CO	Cardiac output
cGMP	Cyclic guanosine 3'-5' monophosphate
cNOS	Constitutive NOS
eNOS	Endothelial nitric oxide synthase
EVLW	Extravascular lung water
EVLWI	Extravascular lung water index
FiO$_2$	Fraction of oxygen in inhaled gas
ICU	Intensive care unit
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
I/R	Ischemia/reperfusion
ITBV	Intrathoracic blood volume
ITTV	Intrathoracic thermal volume
LPS	Lipopolysaccharide
L-NAME	N^G-nitro-L-arginine
LIS	Lung injury score
LV	Left ventricle
NO	Nitric oxide
MB	Methylene blue
MV	Mechanical ventilation
MODS	Multiple organ dysfunction syndrome
nNOS	Neuronal nitric oxide synthase
OA	Oleic Acid
OLV	One lung ventilation
PAP	Mean pulmonary artery pressure
PAOP	Pulmonary artery occlusion pressure
PBVI	Pulmonary blood volume index
PEEP	Positive end expiratory pressure
PE	Pneumonectomy
PPE	Postpneumonectomy pulmonary edema
PVR	Pulmonary vascular resistance
PVRI	Pulmonary vascular resistance index
RV	Right ventricle
TNFα	Tumor necrosis factor alpha
VILI	Ventilator-induced lung injury
VT	Tidal volume
ZEEP	Zero end-expiratory pressure

5. INTRODUCTION

5.1 POSTPNEUMONECTOMY PULMONARY EDEMA IN HUMAN

The first pneumonectomy was performed on a patient with bronchiectasis by the German surgeon R. Nissen in 1931. Two years later, Dr. E.A. Graham carried out the first one-stage pneumonectomy for squamous cell carcinoma. Developed by Drs. W.F. Rienhoff and E.D. Churchill in the 1930s, an individual ligation technique for the hilar structures made lobectomy possible. This intervention was considered a safer and even preferable alternative to pneumonectomy until 1960. However, lung volume reduction surgery is still a complicated and potentially dangerous surgical procedure with major anatomical and physiological challenges per – and postoperatively (Fuentes PA, 2003; Gothard J, 2006).

Postoperative lung injury, also called postpneumonectomy pulmonary edema (PPE), still complicates a significant number of surgical procedures involving lung resection. PPE may develop after pneumonectomy, lobectomy or bilobectomy, although some authors restrict the term only to pulmonary edema following pneumonectomy. Less extensive procedures, such as wedge resections (segmentectomy), are usually excluded. The prevalence of PPE varies between 2.5–14.3% with a mortality rate of 50–100%, which according to recent investigators can be explained by a lack of criteria for early detection of PPE (van der Werff YD *et al.*, 1997; Jordan S *et al.*, 2000; Algar FJ *et al.*, 2003).

The term postpneumonectomy pulmonary edema was used to emphasize its edema-like pattern and a possible relationship to excessive fluid administration following extensive lung resection. More recently, the term acute lung injury (ALI) has been used (Jordan S *et al.*, 2000; Licker M *et al.*, 2003) to highlight the presence of a clinical and histopathological evolution indistinguishable from that seen in ALI and acute respiratory distress syndrome (ARDSnet; Bernard G *et al.*, 1994). Similar to other forms of ALI/ARDS, the presentation of PPE is aggravated by the loss of alveolar and vascular pulmonary tissue. The progression of symptoms and the clinical deterioration challenge even the most appropriate and rapid clinical response.

Clinically, PPE is characterized by acute onset of dyspnea, hypoxemia and radiographic pattern of pulmonary edema together with a rapid irreversible course, despite intensive therapy. The postoperative period in patients with PPE might vary. Different authors report that the onset of respiratory failure may occur during the first 12 h and up to seven days postoperatively (Mathru M *et al.*, 1990; van der Werff YD *et al.*, 1997). However, in most patients PPE develops between the first and the third postoperative day (Turnage WS *et al.*, 1993; Waller DA *et al.*, 1993). Pulmonary artery catheterization or echocardiography may be required in order to exclude a cardiac cause of the pulmonary edema. Admission to an intensive care unit including judicious fluid restriction, proper use of positive pressure ventilation and careful attention to treatment details constitute the best support until a better understanding of the pathogenesis will make more specific therapeutic available for these patients.

In a retrospective study of 10 patients, Zeldin and his co-workers introduced the term PPE after identification of the following risk factors: right-sided pneumonectomy, large volume of perioperative fluids and increased postoperative urine output (Zeldin RA *et al.*, 1984). More recently, the suspicion against a contributory role of excessive fluid volumes has been strengthened and other causative factors have been suggested including the per – and postoperative administration of freshly frozen plasma, damage to the mediastinal lymphatics (Slinger P, 1999), formation and release of cytokines, and toxic oxygen metabolites (Lases EC *et al.*, 2000). Inves-

tigators recently suggested potential risk factors that might contribute significantly to the development of PPE after pulmonary resection (Table 1) (Slinger P, 2002). The significance of these factors will be discussed below.

Table 1
Potential causes of pulmonary edema following pneumonectomy

Probable	Fluid overload
	Lymphatic damage
	Pulmonary capillary pressure changes
	Volume induced lung injury
	Right ventricular dysfunction
	Oxygen toxicity
Possible	Cytokine release

Fluid overload
The findings of Zeldin *et al.* were confirmed in a retrospective study of 243 patients in which PPE occurred with an incidence of 4.5% (Verheijen-Breemhaar L *et al.*, 1988). Other investigators have reported that a 24-h perioperative fluid balance of more than 3 L is associated with a 15 % incidence of PPE and a mortality rate of 43% (Patel RL *et al.*, 1992). However, during the last decade studies have been launched that cast some doubt on a direct relationship between fluid overload and PPE. Thus, investigators reporting the outcome of 402 lung resections found no significant difference in the 24-hour fluid balance between patients with and without PPE (Waller DA *et al.*, 1993). In the largest study including totally 806 pneumonectomies, no differences were found in perioperative fluid balance between patients developing PPE and those taking an uncomplicated course after pneumonectomy (Turnage WS *et al.*, 1993). Other investigators argue that an intraoperative fluid administration of more than 2 L over 4 hours is an independent risk factor for developing PPE (Parquin F *et al.*, 1996), but a positive postoperative fluid balance is not a risk factor *per se*. In a study of five patients after pneumonectomy, the investigators noticed injury to the alveolar - capillary membrane with leaks of high protein content despite a low capillary pressure and normal cardiac output and left ventricular filling pressure (Mathru M *et al.*, 1990). Currently, most investigators believe that perioperative fluid overload is not the primary cause of PPE, but a fluid overload after PE may worsen a lung edema secondary to a rise in pulmonary microvascular permeability. However, it is puzzling to know how much fluids can be given before volume overload might become a problem after a lung has been removed.

Lymphatic damage
The main function of the lung lymphatic system is to clear excessive fluid filtered into the interstitium at the arterial side of the pulmonary microvasculature without being reabsorbed at the venous side. Lung edema occurs when the net amount of filtered fluid into the interstitium exceeds the drainage capacity of the lymphatics. With a normally functioning lymphatic system, lung lymph flow can increase seven to ten-fold in response to the increased filtration forces (Coates G *et al.*, 1984). However, the lymphatic vessels that sample both the ipsilateral and the subcarinal lymph nodes are usually damaged during lobectomy or pneumonectomy. It was found that the lymphatic drainage of the left and the right lung is different (Nohl-Oser HC, 1972). As the right lung is concerned, the lymphatics are for the larger part drained *via* the ipsilateral lymph nodes whereas the left lung is drained via the contralateral lymph vessels. Thus, a right - sided pneumonectomy is more likely associated with PPE than a left-sided. This contention is supported by investigators who found a higher incidence of PPE following right-sided pneumonectomy (Zeldin RA *et al.*, 1984;Turnage WS *et al.*, 1993), whereas others have been unable to con-

firm this finding (Hayes JP *et al.*, 1995; Parquin F *et al.*, 1996). Interestingly, in rabbits obstruction of the lung lymphatic drainage caused a rise in urine production that was prevented after infiltration of the renal parenchyma with the inhibitor of neuronal NOS, 7-nitroindazole (McCormick KM *et al.*, 2004) as well as after administration of the alpha-2-receptor antagonist, prazocin. Whether a similar mechanism exists in human that might prevent the emergence of PPE, is not fully understood.

So, apparently, lymphatic disruption might play a role in the pathophysiology of PPE, particularly after a right-sided PE, but as judged from animal experiments, this mechanism of edema might be partly prevented by renal compensatory responses.

Endothelial damage

A constant finding in patients with PPE is an edema fluid of high protein content (Mathru M *et al.*, 1990) and a histological picture, which is indistinguishable from that seen in patients with ARDS. This may be due to a combination of mechanical and biomolecular effects. Following PE, a hyperdynamic pulmonary circulation has evolved due to single-lung perfusion and ventilation. Increased blood flow velocity can lead to the development of tangential shearing forces, acting on vulnerable points in the microcirculation, such as capillary junctions, which can damage the endothelial barrier (Staub NC, 1978). Similar finding were described by Sinclair et al., who found increased pulmonary vascular permeability throughout the course of ARDS that correlated with increased injury severity score and neutrophil content in BAL fluid (Sinclair DG *et al.*, 1994).

Ventilation

During lung resection, the single ventilated lung may be subjected to a combination of hyperoxia, volutrauma and hyperinflation. The need to increase the inspired oxygen concentration in the contralateral lung to sustain arterial oxygenation during one-lung ventilation may lead to hyperoxia, which represents a potential source of oxidative stress *per se*. Initial ventilator settings with tidal volumes of 10 mL/kg and peak inspiratory pressure of ≤ 30 cmH$_2$O are routinely used, and normally provide adequate ventilation. However, pre-existing lung disease or reduced lung compliance may necessitate the application of a higher tidal volume or peak inspiratory pressure. This might increase mechanical stress to the parenchyma of the remaining lung considerably after a pneumonectomy (Gothard J, 1993).

Following pneumonectomy, a mediastinal shift has been observed accompanied by increased functional residual capacity (Larsson A *et al.*, 1987). A correlation has been shown between the development of PPE and the mediastinal shift (Slinger P, 1999). Even ordinary ventilator settings can produce hyperinflation and volutrauma in such settings. This may result in a direct or indirect trauma to the lung parenchyma. Consistent with the latter findings, Dreyfuss et al. observed a correlation between end-inspiratory volume and the development of volutrauma (Dreyfuss D *et al.*, 1993). Thus, it is close at mind to postulate that the type and intensity of mechanical ventilation during one-lung anesthesia might contribute to the development of PPE. However, to establish a link between ventilator settings and development of PPE warrants further experimental studies.

Right ventricular dysfunction

Dysfunction of the right ventricle (RV) with decreased RV ejection fraction may develop in the postoperative period after pneumonectomy (Reed CE *et al.*, 1992; Kowalewski J *et al.*, 1999). The main reason for this is, most likely, an increase in RV afterload during and after pneumonec-

tomy. RV dysfunction can lead to increased central venous pressure and subsequently to inhibition of lymph clearance from the lungs. This is a consequence of the drainage of thoracic lymph into the left brachiocephalic vein against an elevated central venous pressure, resulting in increased downstream pressure in the lung lymphatics. RV dysfunction can also compromise left ventricle (LV) compliance by ventricular interdependence. Thus, increased LV end-diastolic volume, or filling pressure, will be needed to maintain adequate cardiac output during the postoperative period.

Oxygen toxicity

Evidently, formation of reactive oxygen species (ROS) and oxidative damage plays a role in the onset and progression of ARDS (Pittet JF *et al.*, 1997). This process might be further enhanced by a concomitant decrease in the levels of both primary (Gutterige JM *et al.*, 1994) and secondary antioxidant systems (Cross SE *et al.*, 1990). The formation of pro-oxidant markers appears to depend on the activation of neutrophils, although, particularly in neutropenic patients, other factors should not be ignored (Lamb N *et al.*, 1999). During lung resection, hyperoxic ventilation acting in concert with unfavorable surgical techniques might increase the potential for oxidative stress. In ischemia/reperfusion injury, the investigators noticed increased leakage of ROS from mitochondria concomitant with a depletion of manganese superoxide dismutase (SOD), mainly during re-oxygenation (Ferrari R *et al.*, 1985; Ferrari R *et al.*, 1986; Williams E *et al.*, 1999). When tissues are made ischemic for any length of time, tissue injury ensues (Williams E *et al.*, 1999). In addition, ischemia increases the release and metabolism of arachidonic acid to prostaglandins, with an additional formation and amplification of ROS during lung re-expansion.

Although oxygen toxicity is unlikely to play a major role in the etiology of PPE, it is a factor that can compromise the function of a damaged alveolar-capillary interface. Davis et al. noticed that breathing more than 95% O_2 for 17 hours led to increased plasma albumin in alveolar lavage fluid from human, suggesting that a capillary leak had occurred (Davis WB *et al.*, 1983). The damage following hyperoxic exposure also compromised the ability of the lungs to metabolize biogenic amines, polypeptides, and prostaglandins (Klein J, 1990). Thus, in addition to application of a thorough operation technique, exposure of the remaining lung to a high oxygen concentration should be avoided due to the increased risk of injury to the alveolar-capillary membrane and its effects on the promotion of PPE.

5.2 ANIMAL MODELS OF POSTPNEUMONECTOMY PULMONARY EDEMA

Despite efforts during the last three decades to unveil the etiology and pathogenesis, PPE still remains an elusively defined entity. A variety of mechanisms have been suggested such as ischemia-reperfusion injury, capillary stretching due to increased pulmonary capillary pressure, and volumotrauma secondary to overinflation of the remaining lung. Thus, research by using different animal models to elucidate factors of potential pathogenetic interest may be of great importance.

Studying ischemia-reperfusion in isolated rat lungs, Williams and co-workers, investigated, whether one-lung ventilation (OLV) followed either by reinflation of the collapsed lung or by pneumonectomy might result in lung injury (Williams E *et al.*, 1999). In addition, the authors assessed the effects of ROS and NO in modulating ischemia-reperfusion injury, by using the ROS scavenger superoxide dismutase and the NO synthase inhibitor L-NAME, respectively. Pneumonectomized animals displayed an acute rise in PAP at the time of ligation of the pulmonary vessels, which continued to increase gradually throughout the experiment, but remained unchanged in the control group. Similar findings were also shown in rats (Czartolomna J *et al.*, 1991), dogs (Long JH *et al.*, 1949) and pigs (Roch A *et al.*, 2005; Filaire M *et al.*, 2005).

In animals undergoing OLV, the investigators noticed a fall in the oxygenation index, which is consistent with the findings of authors investigating a model of reperfusion in isolated rat lungs (Matsuzaki Y *et al.*, 1993; Fukuse T *et al.*, 1995). The same effect of pneumonectomy was found in pigs (Roch A *et al.*, 2005). However, in contrast to the latter studies, the experiments carried out by Samano and co-workers showed no decrease in PaO_2/FiO_2 ratio after pneumonectomy (Samano MN *et al.*, 2009). Stable oxygenation values after PE were also reported in a study on anesthetized dogs (Lee E *et al.*, 1985).

A literature search revealed that most researchers agree that PE is followed by an increase in lung microvascular permeability (Jordan S *et al.*, 2000). In isolated rat lungs, extravascular albumin accumulation, which is a marker of vascular injury, was significantly increased in the contralateral lung after lung removal (Williams E *et al.*, 1999). Increased lung capillary permeability and pulmonary edema may also result from an isolated inflammation in which neutrophils play a significant role. Neutrophile accumulation in lung tissue is suggested to be a reliable marker of inflammation and an indirect indication of increased vascular permeability (Sinclair DG *et al.,* 1994). In mice, pneumonectomy subsequently followed by intratracheal instillation of LPS lead to increased number of neutrophils in the lungs and signs of severe histological lung damage (Tajima A *et al.*, 2008). Neutrophile activation also has been noticed after re-expansion of previously collapsed rabbit lung (Funakoshi T *et al.*, 2004). In a study on pigs, histologic examination and measurement of lung myeloperoxidase activity demonstrated that recruitment and activation of neutrophils occurs within the lungs early after PE (Filaire M *et al.*, 2007). In rats undergoing pneumonectomy, Sakuma and co-workers showed that the clearance of alveolar fluid from the remaining lung remained unchanged over the subsequent 7 days (Sakuma T *et al.*, 2002). The latter findings were confirmed by other investigators who noticed no changes in lung microvascular permeability after PE in isolated rabbit lungs (Funakoshi T *et al.*, 2004), and consistently, Samano et al. reported a decrease in lung tissue neutrophil infiltration after PE (Samano MN *et al.*, 2009).

Ischemia/reperfusion (I/R) injury and reactive oxygen species (ROS) formation may contribute to alveolocapillary membrane damage after PE. In a study investigating the effects of I/R on changes in endothelial function and integrity, the authors found that short periods of ischemia followed by reperfusion may cause as much damage as considerably longer periods of ischemia alone. In addition, they observed that HPV in rats was enhanced as compared with baseline values following lung reperfusion, suggesting that release of NO from endothelium might have been impaired (Messent M *et al.*, 1993). In the same model of isolated rodent lungs, Lu et al. found a small rise in extravascular albumin accumulation after ischemia, which increased dramatically if ischemia was followed by reperfusion (Lu YT *et al.*, 1997). Radi et al. administered superoxide dismutase just before reperfusion in a model of isolated lungs. It prevented changes in the vascular control and the permeability to albumin, together with rises in circulating markers of oxidative stress. The authors suggested that superoxide modified the ischemia/reperfusion injury, possibly via its interaction with peroxynitrite, a known initiator of lipid peroxidation generated from the reaction between NO and superoxide anion (Radi R *et al.*, 1991). In a canine model of lung injury, Koyama et al. found that only 6 h of exposure to O_2 caused a marked edema upon reperfusion of isolated lung lobes (Koyama I *et al.*, 1987), however Czartolomna and his co-workers noted that in rats lung isolation and perfusion does not markedly alter pulmonary vascular permeability *per se* (Czartolomna J *et al.*, 1991).

Circulating neutrophils are the potential source of ROS, but neutrophile - depleted rodents seem to respond identical to control animals to I/R-mediated lung injury (Lu YT *et al.*, 1997). Thus, it is unlikely that neutrophile recruitment and activation contribute to immediate onset of

I/R. Other sources of ROS production might be involved in this process. Evidence of hydroxyl radical-like damage was identified in animals after pneumonectomy or OLV followed by lung reinflation, as compared to control animals, subjected to continuous perfusion. In both test groups, reperfusion was accompanied by a maximal rises in plasma markers of ROS damage. Superoxide and NOS inhibitors prevented the formation of hydroxyl-like damage, suggesting that peroxynitrite was formed in this model (Lu YT *et al.*, 1998). In another study the authors suggested that constitutive NOS may act protectively towards I/R injury, supporting the hypothesis that an imbalance between NO production and ROS generation may induce expression of adhesion molecules and local entrapment of neutrophils within the pulmonary capillary bed (Kupatt C *et al.*, 1997).

Imamura et al. reported that the atrial natriuretic peptide (ANP) protects against pulmonary edema in isolated perfused lungs from guinea pigs (Imamura T *et al.*, 1988). However, other investigators have shown that in the acute phase following pneumonectomy in rats, changes in pulmonary ANP and natriuretic peptide receptor expression may contribute to formation of PPE in the remaining lung (Tamura K *et al.*, 2000). The authors reported that ANP concentrations in plasma and lung tissue were higher after pneumonectomy, as compared to the sham operated group. In parallel, in rats expression of natriuretic peptide receptors increased significantly after pneumonectomy. These researchers stated that changes in both natriuretic peptide receptor-C and natriuretic peptide receptor-A expression after PE may prevent PPE in the contralateral lung secondary to an acute increase in the ANP concentration (Tamura K *et al.*, 2000).

5.3 THE RELATIONSHIP BETWEEN PPE AND VENTILATOR-INDUCED LUNG INJURY

In spite of its life-saving role, mechanical ventilation *per se* can result in damage to the lungs, referred to as ventilator- induced lung injury (VILI). During the last decade, VILI has been increasingly paid attention to as a serious complication of mechanical ventilation threatening millions of people worldwide.

In his paper on resuscitation of the apparently dead, John Fothergill suggested that mouth to mouth inflation might be preferable to using a pair of bellows as "the lungs of one man may bear, without injury, as great a force as another man can exert; which by the bellows cannot always be determined" (Whitehead T *et al.*, 2002).

The clinical and radiological manifestations of VILI include pneumothorax, pneumomediastinum and emphysema. The most important mechanisms of VILI include damage of alveolar epithelium and pulmonary vasculature (volumotrauma), release of cytokines and inflammatory mediators (biotrauma), cyclic opening and closing (atelectotrauma) of small airways and lung units and surfactant inactivation as in other forms of lung injury (ARDS), for which mechanical ventilation is an essential treatment (Tsuno K *et al.*, 1991; Gattinoni L *et al.*, 1993; Dreyfuss D *et al.*, 1995; Pittett J *et al.*, 1997; Dreyfuss D *et al.*, 1998). Due to these various reasons, VILI is not limited only to local damage to the airways and the pulmonary vasculature, but through its release of cytokines and pro-inflammatory mediators VILI can result in multiple organ dysfunction syndrome (MODS) and shock (Mandava S *et al.*, 2003).

Delivery of large tidal volumes at high inspiratory pressures may promote the development of volumotrauma, which might progress to VILI (Webb HH *et al.*, 1974; Dreyfuss D *et al.*, 1985; Kolobow T *et al.*, 1987; Tsuno K *et al.*, 1991; Parker JC *et al.*, 1993; Tremblay L *et al.*, 1997). Patients undergoing OLV during general anesthesia are exposed to relatively high tidal volumes (8–10 mL/kg) applied to the dependent lung. In addition, a high inflation pressure is often required. In a study of patients subjected to OLV, the researchers noticed an increase in peak and plateau airway pressures of by 49% and 51%, respectively, immediately upon the commencement of OLV (Szegedi LL *et al.*, 1997). A recent report identified high airway pressure during

surgery as a risk factor for ALI/ARDS. In that work, the investigators found that 42% of the patients with intraoperative peak inspiratory pressures of more than 40 cm H_2O presented with signs of ALI (van der Werff YD *et al.*, 1997). In a dog model, the investigators showed that overinflation of lung tissue resulted in pulmonary edema (Albert RK *et al.*, 1980). According to more recent investigations, intraoperative ventilation with multiple cycles of deflations and reinflations of the dependent lung may lead to lung damage (Williams E *et al.*, 1996; Dos Santos CC *et al.*, 2000).

Short-term mechanical ventilation with tidal volume of 15 mL/kg and ZEEP does not provoke a systemic inflammatory response in patients with healthy lungs (Wrigge H *et al.*, 2000). However, excessive ventilation of patients with concurrent pulmonary disease may induce both a release of cytokines and bacterial translocation from the lungs to the systemic circulation subsequently resulting in cytokine-mediated systemic inflammatory response syndrome (Murphy DB *et al.*, 2000). In rabbit lungs, the response to tracheally instilled endotoxin was investigated under different ventilation modes, protective versus non-protective ventilation. In that study, the non - protectively ventilated group was characterized by elevated plasma levels of endotoxin and TNF-α, with a fall in PaO_2 and MAP. However, other authors found no differences in cytokine release between protective and non-protective lung ventilation strategies during thoracic surgery within 3 hours (Wrigge H *et al.*, 2004).

Studies performed on isolated lungs as well as on intact animals have shown that injured lungs are more susceptible to VILI (Dreyfuss D *et al.*, 1995). Acute inflammation is a characteristic of all types of pulmonary surgery, and the degree of trauma is crucial for the severity of the inflammatory response and the amount of mediator release (Yamada T *et al.*, 1998; Wrigge H *et al.*, 2004). In patients with malignancies, cytokines that were activated prior to surgery increased further postoperatively (Waller DA *et al.*, 1996; Inada K *et al.*, 2000; Craig SR *et al.*, 2001). Consistently, by using a rabbit model of re-expansion pulmonary edema, Sakao and co-workers found cytokine activation in association with the development of lung damage (Sakao Y *et al.*, 2001).

Experiments *in vivo* have suggested that activation of the NO system is involved in the pathogenesis of VILI (Preiser JC *et al.*, 2001; Choi WI *et al.*, 2003; Broccard AF *et al.*, 2004; Peng X *et al.*, 2005; Liaudet L *et al.*, 2008). Stromberg and co-workers found that NO excretion increased subsequent to enhancement of end-expiratory volumes (Stromberg S *et al.*, 1997). Briefly, the authors examined how different types of ventilation or changes in CO_2 affect the formation of endogenous NO. The animals were enclosed in a chamber and subjected to various modes of positive as well as negative pressure ventilation. Application of both negative and positive end-expiratory pressure produced similar increase in NO production. Thus, mechanical stretch of lung tissue, *per se,* can enhance endogenously produced NO.

An alternative way of activating the L-arginine/NO system during excessive ventilation is by transmission of bacteria through the overstretched alveolar epithelium, with subsequent activation of the systemic inflammatory response syndrome. This includes activation of *i*NOS, which increases endogenously produced NO (Feihl F *et al.*, 2001; Lachmann RA *et al.*, 2007). After reaction with superoxide to generate the highly reactive free radical peroxynitrite, the cytotoxic effects of NO increases. This reaction is supposed to play an important role in the pathogenesis of multiple organ dysfunction syndromes (MODS) that might be the ultimate outcome of VILI (Liadet L *et al.*, 2000).

In thoracic surgery, patients undergoing lung removal are at high risk of developing PPE. In these patients VILI is an apparent threat. On the other hand, careful handling of the ventilatory settings alone, particularly, a reduction of the tidal volume from 12 to 6 mL/kg has been shown

to increase survival from ARDS by 22% (The ARDS network, 2003). The VILI concept has been supported by numerous experimental studies. However, further research is necessary to fully elucidate the interaction between VILI and postpneumonectomy pulmonary edema.

5.4 AN OVINE MODEL OF POSTPNEUMONECTOMY PULMONARY EDEMA

Since VILI and PPE has been reproduced mostly in small experimental animals and isolated lungs, the results of these studies might have limited clinical applicability (Peevy KJ *et al.*, 1990). Moreover, the major differences in the course of pathophysiological changes have been shown to depend on the size of animals (Richard JD *et al.*, 2003).

Sheep has been used for almost four decades to model ALI from exposure to endotoxin. Importantly, sheep is a large animal of a size which is comparable with man. Thus, as lung pathophysiology is concerned, findings made in sheep of 40-60 kg body weight, appear to be clinically more relevant than those performed in small laboratory animals.

It is difficult to provoke VILI in the lungs of large animals. Thus, Garcia-Delgado et al. found no signs of pulmonary edema in healthy pigs after being subjected to mechanical ventilation with VT 50 mL/kg over 4 hours (Garcia-Delgado M *et al.*, 2006). On the opposite, Mandava et al. in a study on sheep subjected to mechanical ventilation at high inflation pressures observed pneumothorax and death after 18 hrs in nearly half of the animals (Mandava S *et al.*, 2003). In contrast to small animals, that developed reproducible pulmonary edema when exposed to high VT, large animals tend to respond with barotrauma on mechanical ventilation with high volumes and pressures. However, acute inflammatory injury occurs in all types of pulmonary surgery. Thus, apparently, the lungs are more susceptible to injury in the postoperative period.

In 1985, Lee et al. established a pneumonectomy model of pulmonary edema in dogs (Lee E *et al.*, 1985). The main goal of their work was to find out whether animals are more susceptible to accumulation of EVLW after PE at various left heart filling pressures. To evaluate EVLW, the authors used a double indicator technique described by Lewis and co-workers (Lewis FR *et al.*, 1978). However, the investigators concluded that pneumonectomy does not acutely increase the susceptibility to accumulation of EVLW upon elevations of the left heart filling pressure. Provided capillary permeability and oncotic pressure are stable, increments in EVLW are related to increases in left heart pressures. According to the latter workers, this tendency did not worsen shortly after the pneumonectomy because the lymphatics of the remaining lung were able to function effectively enough to keep the interstitium clear. In contrast, investigators who administered fluid at an amount that was sufficient to raise the left atrial pressure to 23 mm Hg noticed a difference in EVLW before and after pneumonectomy. In their experiments, the fluid challenges produced significant changes in Hct and total protein content (Little AG *et al.*, 1984). These findings were consistent with Guyton et al., who showed that both elevated left heart and microvascular pressures together with decreased plasma oncotic pressures might rapidly overwhelm the lymphatic clearance capacity of the remaining lung and invoke the formation of pulmonary edema (Guyton AC *et al.*, 1959). Lately, Roch et al., found increased EVLWI and decreased PaO_2/FiO_2 ratio accompanied by increased shunting and increased PAP after PE and subsequent oleic acid injection in pigs (Roch A *et al.*, 2005). The authors concluded that EVLWI measurements may be a useful tool in early recognition of respiratory failure after pneumonectomy in clinical settings.

To conclude, postpneumonectomy pulmonary edema is a dangerous type of ALI, caused by a variety of reasons, and mechanical ventilation remains an indispensable supportive tool in these patients. However, despite its life-saving role MV can result in ventilator induced lung injury. Since it has been reproduced mostly in small intact experimental animals, it is still unsettled if PPE in large animals might be triggered by ventilation with excessive tidal volumes combined

with ZEEP. Moreover, the involvement of the L-arginine/NO pathway in the pathogenesis of PPE and the efficacy of different NOS inhibitors in preventing this condition might be the subject of further investigations.

6. AIMS OF THE STUDY

The main objectives of these studies were to find out in anesthetized sheep if PPE is triggered by an injurious type of ventilation including excessive tidal volumes in combination with zero end expiratory pressure. Furthermore, we speculated whether increased generation of NO is involved in the lung injury and whether inhibitors of NO synthase would antagonize the emergence of PPE.

The specific aims were as follows:

1. To explore the evolution of postpneumonectomy edema after injurious ventilation of the remaining lung by specifically focusing on changes in arterial oxygenation, lung hemodynamics and extravascular lung water (Paper I).
2. To study the effect of treatment with intravenously infused methylene blue (MB; methylthionine) on blood gases and volumetric and hemodynamic variables, with special emphasis on extravascular lung water (Paper II).
3. To investigate the effects of intravenous administration of the inhibitor of neuronal nitric oxide synthase (*n*NOS), 7-nitroinidazole, on lung morphology, gas exchange, metabolic, hemodynamic and volumetric variables (Paper III).

7. METHODS

7.1 THE ANIMAL MODEL

7.1.1 ETHICS
The experiments were approved by the University of Tromsø committee on research animal ethics under the Norwegian National Animal Research Authority in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Convention No. 123 Issued by the Council of Europe).

7.1.2 SPECIES
The experiments were conducted on 75 yearling sheep weighing 38.0 ± 5.8 kg (mean $\pm$ SD). Body surface area was calculated as $BW^{0,67}$ x $0,084$, where BW is body weight in kilograms.

7.1.3 ANESTHESIA
All sheep underwent instrumentation and experimentation under general anesthesia.

PAPER I. After injection of thiopental sodium 20 mg/kg (Pentothal Natrium®, Abbott, North Chicago, IL, USA), the animals were intubated, placed in the lateral position, and conventionally ventilated in volume-controlled mode (FiO_2 0.5; tidal volume 6 mL/kg; PEEP 2 cm H_2O). General anesthesia was maintained with a continuous infusion of ketamine hydrochloride (Ketalar®, Parke–Davis, Solna, Sweden), midazolam (Dormicum®, F. Hoffman–La Roche AG, Basel, Switzerland), and fentanyl (Fentanyl®, Hamelin pharma group, Hamelin, Germany) at rates of 3.0 mg/kg/hr, 0.4 mg/kg/hr, and 12 μg/kg/hr, respectively. Muscle relaxation was induced with pancuronium bromide 0.1 mg/kg as an initial bolus followed by infusion of 0.06 mg/kg/hr after confirmation of pain-free anesthesia. Throughout the experiments, all the animals received an infusion of lactated Ringer's solution at a constant rate of 8 mL/kg/hr. Special attention was paid to protecting the airways against contamination with gastric contents by inserting a gastric tube.

PAPER II-III. Anesthesia was induced with thiopental sodium 15–20 mg/kg (Pentothal Natrium®, Abbott, North Chicago, IL, USA) and maintained with a combination of ketamine hydrochloride 3 mg/kg/hr (Ketalar®, Parke–Davis, Solna, Sweden), midazolam 0.4 mg/kg/hr (Dormicum®, F. Hoffman–La Roche AG, Basel, Switzerland), and fentanyl 12 μg/kg/hr (Fentanyl®, Hamelin pharma group, Hamelin, Germany). Sheep were ventilated with a Servo*i* Universal respirator (Maquet Critical Care AB, SOLNA, Sweden) using a volume controlled mode with tidal volume (VT) 6 mL/kg, FiO_2 0.5, inspiration:expiration ratio 1:2 and positive end-expiratory pressure (PEEP) 2 cm H_2O. To keep $PaCO_2$ values within the normal range we connected a plastic bottle and additional tubes in the breathing circuit to increase apparatus dead space, as necessary. Body temperature was maintained at 38 °C.

7.1.4 INSTRUMENTATION
PAPER I. The external jugular vein and the femoral artery were cannulated using standard introducers (8.5F, I350BF85, Edwards Lifesciences, Irvine, CA, USA). A thermodilution pulmonary artery catheter (7.0F, F131HF7; Edwards Life Sciences, Irvine, CA, USA) was introduced into the pulmonary artery. A fiberoptic thermistor-tipped thermal-dye dilution catheter (4F PULSIO-CATH: PV2024L; Pulsion Medical Systems, Munich, Germany) was inserted *via* a femoral artery through arterial introducers into the abdominal aorta and connected to a COLD Z-021 monitor (Pulsion Medical Systems, Munich, Germany). Correspondingly, an additional thermistor-tipped thermodilution catheter (PV2014L50LGW) was placed in the abdominal aorta via a sec-

ond introducer in the same femoral artery and connected to a PiCCO*plus* monitor (Pulsion Medical Systems, Munich, Germany) in order to compare the accuracy and efficacy of the two techniques for determination of extravascular lung water.

The fiberoptic thermal-dye dilution catheter and the pulmonary artery catheter were continuously flushed with a solution of heparin in NaCl 0.9% (5 IU/kg/hr) and connected to standard pressure transducers (Transpac®III, Abbott, North Chicago, IL, USA). Left or right-sided lateral thoracotomy was performed in the 5th intercostal space. After dissection of the mediastinal pleura, the pulmonary root was ligated using a cotton band fixed with a clamp. This technique substantially reduced the time of the procedure and the blood loss. Then, the lungs were separately removed and prepared for *ex vivo* determination of extravascular lung water by gravimetry.

PAPER II-III. A 7F flow-directed pulmonary artery catheter (131HF7; Baxter, Irvine, CA, USA) was placed in the pulmonary artery and a 4F fiberoptic thermo–dye dilution catheter (PV2024L, Pulsion Medical Systems, Germany) was inserted *via* an arterial introducer (Super Arrow-Flex PSI Set, CP-07511, Arrow, Reading, PA, USA) into the aortic arch. Both catheters were connected to pressure transducers (Transpac III; Abbott, North Chicago, IL, USA) and flushed with a solution of heparin 10 IU/kg/h (Heparin, LEO Pharma AS, Ballerup, Denmark). We performed a right-sided pneumonectomy *via* the fifth intercostal space, using the same surgical techniques as described for Paper I. The animals received Ringer's acetate at a rate of 10 mL/kg/hr intravenously throughout the experiments.

7.2 MEASUREMENTS

7.2.1 HEMODYNAMIC AND VOLUMETRIC VARIABLES

PAPER I. Consists of two parts: a study aimed at assessing the accuracy of the PiCCO*plus* monitor for determination of extravascular lung water after pneumonectomy, and a study of injurious ventilation after pneumonectomy. The first part of this paper was presented in a previous dissertation (V. Kuzkov, 2007). Thus, in the present thesis we investigated the significance of excessive tidal volumes as a part of the pathogenesis of postpneumonectomy pulmonary edema. All volumetric parameters in this thesis were evaluated by the thermo–dye dilution technique using COLD Z-021 (Pulsion Medical Systems, Germany).

All the variables were registered at baseline, after lateral thoracotomy, shortly after pneumonectomy, and at 1 hr after pneumonectomy. In the injurious ventilation study, which is the topic of this thesis, the protocol was expanded, and additional measurements were performed at 2 and 4 hrs after pneumonectomy in both the INJV and the PROTV groups.

Mean arterial pressure, pulmonary arterial pressure, pulmonary artery occlusion pressure, and right atrial pressure were displayed on a Patient Data Monitor (565A, Kone, Espoo, Finland) and recorded by a Gould 6600 Polygraph (Gould Instruments, Cleveland, OH).

Volumetric variables were calculated as an average of triplicate bolus injections of ice-cold solutions of indocyanine green (1 mg/mL, 6 mL) and dextrose (5%, 10 mL) for TDD and STD, respectively. These indicator solutions were injected into the right atrium randomly during the respiratory cycle. Systemic and pulmonary vascular resistance indexes were calculated using standard equations (Kirov MY *et al.*, 2004). Cardiac index (CI) was registered in the pulmonary artery and in the aorta. Using TDD and STD, we determined EVLW index (EVLWI), intrathoracic blood volume index (ITBVI), intrathoracic thermal volume index (ITTVI), and global end diastolic volume index (GEDVI). Pulmonary blood volume index was measured directly by TDD (PBVI$_{TDD}$) only. We calculated the true relationship between ITBVI and GEDVI by using their directly measured values as determined with TDD. In addition, pulmonary vascular perme-

ability indexes were calculated as $PVPI_{PBV} = EVLWI_{TDD}/PBVI_{TDD}$ and $PVPI_{ITBV} = EVLWI_{TDD}/ITBVI_{TDD}$ (Kofidis T *et al.*, 2003; Matejovic M *et al.*, 2004).

PAPER II-III. Hemodynamic and volumetric variables were determined at baseline, after lateral thoracotomy, 15 min after PE (time 0 hrs) and thereafter hourly until the end of the experiment. All the thermodilution variables were computed as a mean of 3 measurements, each consisting of an 8 ml bolus of 1% indocyanine green in ice-cold sterile water (thermal-dye dilution) injected into the right atrium. Extravascular lung water index (EVLWI), pulmonary vascular permeability index (PVPI), cardiac index (CI), cardiac function index (CFI), pulmonary blood volume index (PBVI), global end-diastolic volume index (GEDVI), intrathoracic blood volume index (ITBVI) and left heart end diastolic volume index (LHEDVI) were assessed by thermo–dye dilution using a COLD Z-021 monitor (Pulsion Medical Systems, Germany). The mean systemic artery pressure, mean pulmonary artery pressure (PAP), pulmonary artery occlusion pressure (PAOP), heart rate (HR) and mean right atrial pressure were displayed on a 565A Patient Data Monitor (Kone, Espoo, Finland) and stored in Lab View (National Instruments Corporation, Austin, TX). In addition, the systemic vascular resistance index (SVRI) and the pulmonary vascular resistance index (PVRI) were calculated.

7.2.2 GAS EXCHANGE AND VENTILATION MECHANICS

PAPER I-III. Blood gas variables were determined hourly. Samples were drawn from the systemic (a) and the pulmonary (v) arteries and analyzed for pH, PCO_2, HCO^{3-}, PO_2, SO_2, hemoglobin, and hematocrit (Rapid 860, Chiron Diagnostics Corporation, East Walpole, MA). Alveolar-arterial oxygen tension difference ($AaPO_2$), PaO_2/FiO_2 ratio and venous admixture (Qs/Qt) were calculated using standard equations hourly (Evgenov OV *et al.*, 2001). Peak airway pressure (P_{Peak}) and airway plateau pressure ($P_{Plateau}$) and PEEP were monitored by the Servo ventilator. Total lung and chest quasistatic compliance (C_{QS}) was calculated as: $C_{QS} = VT/(P_{Plateau} - PEEP)/Body weight (kg)$.

7.2.3 PLASMA CONCENTRATIONS OF NITRITES AND NITRATES

PAPER II. Plasma concentrations of nitrites and nitrates (NO_2/NO_3) were determined at the baseline and at the end of experiment using a nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI).

PAPER III. Samples were taken at baseline, at time 0 hrs and at the end of experiment, and analyzed using Cayman nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA).

7.2.4 EUTHANASIA

PAPER I-III. After completion of the experiments, all the sheep were sacrificed with an intravenous injection of pentobarbital (Pentobarbital® NAF, Ås Production Lab, Ås, Norway) 100 mg/kg followed by 50 mmol KCl (KCl® NAF, Ås Production Lab, Ås, Norway).

7.3 LUNG SAMPLING AND HISTOLOGIC EXAMINATION (PAPER III)

In five sheep from each group, a histological lung injury score (LIS) was determined by using a modified version of the method proposed by Zhou and colleagues (Zhou ZH *et al.*, 2000), as previously reported from our group (Waerhaug K *et al.*, 2008). Representative tissue blocks from the upper and lower lobes were preserved in 4% formaldehyde, sectioned and stained with haematoxylin and eosin. A pathologist without knowledge of the group identity examined the sections by light microscopy.

Lung edema was assessed separately as the severity of subpleural, interlobular and interalveolar edema by measuring the thickness of pleura, interlobular and interalveolar septa in relation to the diameter of the average alveolar space, expressed in per cent. The arithmetic mean of the percentage {subpleural edema + interlobular edema + interalveolar edema/3 (%)} was used as a general interstitial edema coefficient. The interstitial edema score was set to 0 when the percentage general interstitial edema coefficient was below 30, and correspondingly, to 1 when general interstitial edema coefficient was between 31 and 60, to 2 when general interstitial edema coefficient was between 61 and 100, and to 3 when general interstitial edema coefficient exceeded 100.

Leukocyte infiltration was calculated as the average number of neutrophil granulocytes per high power field (HPF, 0.1734 mm^2). A minimum of 10 HPFs were assessed per slide. The leukocyte infiltration score was set to 0 when average number of neutrophil granulocytes was 0-2; 1 when ANNG was 3–10; 2 when average number of neutrophil granulocytes was 11–40 and 3 when average number of neutrophil granulocytes was 41 or higher.

Intra-alveolar hemorrhage was first assessed as the number of blood-containing alveoli per 10 alveoli. Then, an average intra-alveolar volume of hemorrhage was estimated and expressed in percent. A minimum of 30 alveoli was assessed. Finally, we calculated the absolute hemorrhage volume coefficient per alveolus by multiplying the number of affected alveoli with the average intra-alveolar volume of hemorrhage and then dividing the product by 10. The hemorrhage score was 0 when the absolute hemorrhage volume coefficient was 0–0.5; 1 when the absolute hemorrhage volume coefficient was 0.51–2; 2 when the absolute hemorrhage volume coefficient was 3–10 and 3 when the absolute hemorrhage volume coefficient was 11 or higher.

Hyaline membranes were calculated per 10 alveoli and a minimum of 30 alveoli per slide was assessed to determine the average quantity of hyaline membrane. Hyaline membrane score was estimated as 0 when the average quantity of hyaline membrane was 0; 1 when the average quantity of hyaline membrane was 1–2; 2 when the average quantity of hyaline membrane was 3–5 and 3 when the average quantity of hyaline membrane was 6–10. Additionally, we calculated the absolute bronchial desquamation volume coefficient in a way similar to that for the absolute hemorrhage volume coefficient .

The desquamation score was 0 when the absolute bronchial desquamation volume coefficient was 0–1; 1 when the absolute bronchial desquamation volume coefficient was 2–5 and 2 when the absolute bronchial desquamation volume coefficient was 6–10) and the percentage of alveoli involved in atelectasis. The morphology was performed in a blinded manner by a trained pathologist not being able to identify the treatment and outcome of the animals until completion of the study.

Typical photomicrographs were taken using a Leica DM 2500 microscope and a Leica DFC 320 digital camera, with the software Leica IM50 (Leica Microsystems GmbH, Wetzlar, Germany).

7.4 EXPERIMENTAL PROTOCOLS

7.4.1. PAPER I
The study included two parts: a pneumonectomy study (n=18) and an injurious ventilation study (n=12). The aim of the study was twofold; firstly, to evaluate the accuracy of the single thermodilution and the thermo–dye dilution methods in comparison with postmortem gravimetry for measurement of EVLW after pneumonectomy; and secondly, to explore the role of ventilator-induced lung injury in the pathogenesis of postpneumonectomy pulmonary edema. Evaluation of

the accuracy of single thermodilution versus the thermo–dye dilution in relation to pneumonectomy was the issue of a previous dissertation, and is beyond the scope of this thesis.

In the injurious ventilation part of the study all the sheep (n = 12; weight, 36.0 ± 4.5 kg), were subjected to right pneumonectomy and randomly assigned to an injuriously ventilated group (INJV, n = 6), that was ventilated with a tidal volume (VT) of 12 ml/kg and zero end-expiratory pressure (ZEEP) or to a protectively ventilated group (PROTV, n = 6) that was ventilated with VT of 6 ml/kg and positive end-expiratory pressure (PEEP) of 2 cm H_2O. Both groups were ventilated with these tidal volumes until cessation of the experiment 4 hrs after pneumonectomy.

7.4.2. PAPER II
After PE, the sheep were randomized either to a protectively ventilated group with VT 6 mL/kg, FiO_2 0.5, I:E ratio 1:2, and PEEP of 2 cm H_2O throughout the experiment (PROTV group; n = 7) and two groups ventilated with VT 12 mL/kg, FiO_2 0.5, I:E ratio 1:2, and ZEEP, one control ("injurious ventilation"; INJV group; n = 7) and one which was treated after one hour of injurious ventilation with an intravenous administration of 3 mg/kg of methylene blue (MB) (Methyl-thionine®; Nycomed, Oslo, Norway) over 15 minutes, followed by a continuous infusion at a rate of 3 mg/kg/hr throughout the remaining 3 hrs of the experiment (INJV+MB group; n = 7).

7.4.2. PAPER III
After baseline measurements, thoracotomy and pneumonectomy, all the animals were randomly assigned to one of the following groups:

1. Protective ventilation (PROTV; n= 8); with VT 6 ml kg^{-1}, FiO_2 0.5, respiratory rate (RR) 25–27 1 min^{-1}, I:E ratio 1:2, and PEEP 4 cm H_2O;

2. Injurious ventilation (INJV; n = 8) throughout the 8 hour post-pneumonectomy period with (VT 12 ml kg^{-1}, FiO_2 0.5, RR 12–13 1 min^{-1}, I:E ratio 1:2, and PEEP 0 cm H_2O);

3. Injurious ventilation, as above, with subsequent administration of the inhibitor of neuronal nitric oxide synthase (nNOS) 7-nitroindazole (N7778, 7-Nitroindazole; Sigma-Aldrich, St. Louis, MO, USA) 1 mg/kg/hr dissolved as described by the manufacturer, added to the remainder of Ringer's acetate and infused intravenously from 2 hours after commencement of injurious ventilation and throughout the remaining 6 hrs of experiment (INJV+NI; n = 8). The INJV and the PROTV groups received the solvent dissolved in the Ringer solution only.

7.5 STATISTICS

Statistical analysis was performed using the SPSS 13.0/15.0 software package (SPSS, Chicago, IL, USA). For each continuous variable the normality of distribution was checked using the Shapiro-Wilk (Paper I) or Kolmogorov-Smirnov test (Paper II-III). If a normal distribution could not be demonstrated, such as for the lung injury score, the Kruskal–Wallis test was used to detect differences between the groups (Paper III). Continuous data were expressed as mean ± SD (Paper I-III) and assessed for intragroup and intergroup differences by two-way analysis of variance (ANOVA) followed by post hoc Scheffe's test (Paper I-III). For comparison of two groups of independent variables, we used the Mann-Whitney U test (Paper I). P value of < 0.05 was considered as statistically significant.

8. SYNOPSIS OF THE RESULTS

8.1. PAPER I: EXTRAVASCULAR LUNG WATER AFTER PNEUMONECTOMY AND ONE-LUNG VENTILATION IN SHEEP

Paper I is a shared paper, which was also included in the thesis of the first author. The study had two aims; the first was to evaluate two methods for measuring extravascular lung water against post mortem gravimetry in anesthetized sheep; the second was to study the effects of injurious ventilation after pneumonectomy. In the injurious ventilation part of the study, we investigated whether or not the evolution of post pneumonectomy lung edema was dependent on aggressive ventilation of the remaining lung.

After uncomplicated right-sided pneumonectomy, we noticed significant increments in PAP and PVRI, but no changes in HR, MAP, CI, or SVRI (Table 1). When the pneumonectomy was followed by injurious ventilation RAP, PAP, PAOP, and PVRI all increased ($p < 0.05$). In addition, after pneumonectomy both ITBVI and GEDVI reduced by 11-29% ($p < 0.05$).

After pneumonectomy, $EVLWI_{STD}$ and $EVLWI_{TDD}$ decreased both in the INJV ($p < 0.02$) and the PROTV ($p < 0.05$) groups (Fig. 1). In the INJV group, $EVLWI_{STD}$ and $EVLWI_{TDD}$ primarily decreased by 40.3% ± 1.7% and 43.1% ± 13.7%, respectively, compared with baseline. Correspondingly, in the PROTV group, $EVLWI_{STD}$ and $EVLWI_{TDD}$ decreased by 38.7% ± 9.9% and 43.1% ± 14.3%, respectively ($p < 0.05$). After 4 hrs of injurious ventilation, we observed significant elevations in both $EVLW_{STD}$ and $EVLWI_{TDD}$ as compared with the values after pneumonectomy ($p < 0.001$ and $p < 0.02$, respectively). In contrast, pneumonectomy followed by protective ventilation did not result in significant changes in EVLW indexes. By 4 hrs after pneumonectomy, both $EVLWI_{STD}$ and $EVLWI_{TDD}$ were significantly higher in the INJV group.

After pneumonectomy, $PBVI_{TDD}$ decreased both in the PROTV and the INJV groups. In parallel, injurious ventilation was associated with increased $ITBVI_{STD}$, $PVPI_{PBV}$, and $PVPI_{ITBV}$ ($p < 0.05$) as compared with the PROTV group.

In the INJV group, PaO_2 decreased and Q_S/Q_T increased significantly after the thoracotomy. After the pneumonectomy, airway pressures increased substantially and C_{QS} decreased in both groups; however, these changes were more prominent in the INJV group ($p < 0.05$). In addition, at 4 hrs after pneumonectomy, the INJV group showed a significant increase in central temperature, hematocrit, and hemoglobin concentration as compared with the PROTV group (data not shown).

Table 1. Changes in volumetric, hemodynamic, and respiratory variables in sheep exposed to injurious ventilation.

Parameter	Group	Measurement points					
		Baseline	Thoracotomy	PE	1 hr after PE	2 hrs after PE	4 hrs after PE
ITBVI$_{TDD}$, mL/m^2	INJV	895 ± 133	854 ± 139	739 ± 159	798 ± 125	804 ± 132	762 ± 96
	PROTV	806 ± 131	793 ± 192	678 ± 112	680 ± 148	713 ± 102	682 ± 109
PBVI$_{TDD}$, mL/m^2	INJV	280 ± 63	230 ± 34	166 ± 49 [a]	182 ± 50	184 ± 42	174 ± 51 [a]
	PROTV	261 ± 50	231 ± 55	175 ± 34 [a]	187 ± 45	196 ± 37	178 ± 30 [a]
ITBVI$_{STD}$, mL/m^2	INJV	700 ± 129	735 ± 94	614 ± 98	655 ± 92	672 ± 73 [c]	649 ± 94
	PROTV	700 ± 137	646 ± 92	543 ± 90	548 ± 70	571 ± 55	600 ± 84
ITBVI/GEDVI, rel.	INJV	1.46 ± 0.09	1.38 ± 0.10	1.29 ± 0.04 [a, c]	1.29 ± 0.05 [a, c]	1.29 ± 0.03 [a, c]	1.30 ± 0.09 [a]
	PROTV	1.39 ± 0.15	1.42 ± 0.07	1.45 ± 0.17	1.37 ± 0.04	1.41 ± 0.07	1.37 ± 0.05
PVPI$_{PBV}$, rel.	INJV	1.05 ± 0.44	1.36 ± 0.49	1.02 ± 0.47	1.07 ± 0.77	1.29 ± 0.66 [c]	1.92 ± 0.77 [c]
	PROTV	0.93 ± 0.25	1.29 ± 0.31	0.79 ± 0.29	0.66 ± 0.20	0.66 ± 0.21	0.71 ± 0.25
PVPI$_{ITBV}$, rel.	INJV	0.32 ± 0.11	0.36 ± 0.12	0.22 ± 0.09	0.22 ± 0.12	0.28 ± 0.12 [c]	0.40 ± 0.09 [c]
	PROTV	0.31 ± 0.09	0.38 ± 0.11	0.20 ± 0.05	0.18 ± 0.06	0.18 ± 0.05	0.19 ± 0.06
PAP, mm Hg	INJV	12 ± 2	12 ± 2	16 ± 3	18 ± 4	23 ± 6 [a]	29 ± 7 [a, b, c]
	PROTV	12 ± 2	12 ± 3	16 ± 2	17 ± 4	17 ± 3	16 ± 3
PVRI, dyn×sec/cm^5/m^2	INJV	135 ± 62	112 ± 50	206 ± 146	254 ± 160	321 ± 113 [c]	461 ± 138 [a, b, c]
	PROTV	121 ± 26	148 ± 43	270 ± 71 [a]	214 ± 42 [a]	213 ± 24 [a]	213 ± 40 [a, b]
PaO$_2$/FiO$_2$, mm Hg	INJV	323 ± 30	257 ± 54 [c]	214 ± 41 [a, c]	225 ± 49 [a, c]	196 ± 41 [a, c]	117 ± 36 [a, b, c]
	PROTV	394 ± 82	372 ± 85	321 ± 50	370 ± 79	399 ± 67	396 ± 78
Q$_S$/Q$_T$, %	INJV	2.8 ± 0.8	3.6 ± 1.9	3.3 ± 0.5	3.5 ± 1.4	4.3 ± 2.9	27.8 ± 14.8 [a, b, c]
	PROTV	2.0 ± 1.1	2.5 ± 0.5	2.5 ± 1.0	2.3 ± 0.5	2.1 ± 0.8	2.2 ± 1
Ppeak, cm H$_2$O	INJV	20.7 ± 2.8	20.5 ± 3.7 [b]	36.6 ± 3.7 [a]	45.8 ± 4.5 [a, b, c]	48.7 ± 4.5 [a, b, c]	50.0 ± 1.5 [a, b, c]
	PROTV	19.1 ± 3.9	16.4 ± 3.0 [b]	37.0 ± 6.7 [a]	34.1 ± 6.3 [a]	34.3 ± 6.1 [a]	35.1 ± 6.7 [a]
Pplat, cm H$_2$O	INJV	17.0 ± 3.9	16.0 ± 5.0 [b]	27.3 ± 4.1 [a]	36.4 ± 4.7 [a, c]	43.1 ± 7.3 [a, b, c]	44.6 ± 4.4 [a, b, c]
	PROTV	16.5 ± 4.3	13.3 ± 2.6 [b]	29.3 ± 4.2 [a]	26.4 ± 3.0 [a]	26.8 ± 5.4 [a]	26.7 ± 6.9 [a]
C$_{QS}$, mL/cm H$_2$O/kg	INJV	0.49 ± 0.12	0.48 ± 0.13 [b]	0.26 ± 0.06 [a]	0.34 ± 0.05 [c]	0.30 ± 0.06 [a, c]	0.27 ± 0.06 [a]
	PROTV	0.43 ± 0.10	0.55 ± 0.14 [b]	0.22 ± 0.02 [a]	0.24 ± 0.03 [a]	0.24 ± 0.04 [a]	0.25 ± 0.08 [a]

Data presented as mean ± SD. PE, pneumonectomy; INJV, injurious ventilation group; PROTV, protective ventilation group; PE, pneumonectomy; ITBVI$_{TDD}$, intrathoracic blood volume index determined by thermal-dye dilution; PBVI$_{TDD}$, pulmonary blood volume index determined by thermal-dye dilution; ITBVI$_{STD}$, intrathoracic blood volume index determined by single thermodilution; rel., relative value; PVPI$_{PBV}$, pulmonary vascular permeability index determined as EVLWI$_{TDD}$/PBVI$_{TDD}$; PVPI$_{ITBV}$, pulmonary vascular permeability index determined as EVLWI$_{TDD}$/ITBVI$_{TDD}$; PAP, pulmonary artery pressure; PVRI, pulmonary vascular resistance index; PaO$_2$, arterial partial oxygen pressure; FiO$_2$, fraction of inspired oxygen; Q$_S$/Q$_T$, venous admixture; Ppeak, peak airway pressure; Pplat, plateau airway pressure; C$_{QS}$, total lung and chest quasistatic compliance.
[a] $p < 0.05$ within the groups compared with baseline; [b] $p < 0.05$ within the groups compared with pneumonectomy; [c] $p < 0.05$ between groups in Mann-Whitney U-test
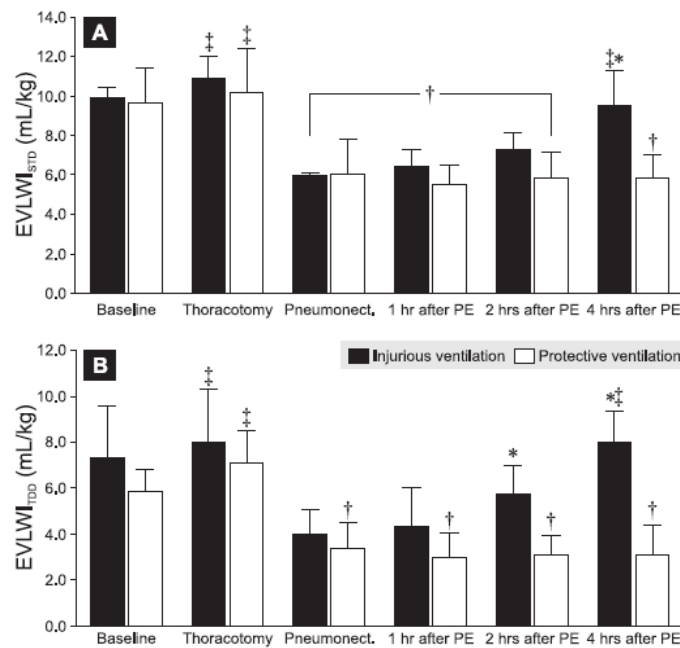
Figure 1. Changes in extravascular lung water index (EVLWI) determined with single thermodilution (*Panel A*) and thermal-dye dilution (*Panel B*) in sheep subjected to pneumonectomy followed by injurious ventilation of the residual lung.
Data are presented as mean ± SD. $EVLWI_{TDD}$, extravascular lung water index determined with thermal-dye dilution; $EVLWI_{STD}$, extravascular lung water index determined with single thermodilution.
* $p < 0.05$ between the groups; † $p < 0.05$ within the group compared with baseline; ‡ $p < 0.05$ within the group compared with pneumonectomy.

8.2. PAPER II: THE EFFECTS OF METHYLENE BLUE ON OVINE POST-PNEUMONECTOMY PULMONARY EDEMA

Our aim was to determine, in anesthetized sheep, whether treatment with MB would resist the rise in EVLW, the decrease in arterial oxygenation and the enhanced generation of NO after lung injury induced by a combination of PE, followed by ventilation with excessive tidal volumes and ZEEP.

Pneumonectomy reduced pulmonary blood volume, EVLWI (Fig. 2) and quasistatic lung compliance in all groups, in parallel with a rise in peak airway pressure (P<0.05). In the INJV group, pulmonary arterial pressure, EVLWI and pulmonary vascular permeability index increased, and arterial oxygenation decreased towards cessation of the experiments. These changes were not antagonized by MB. Plasma NOx increased in all the groups compared with baseline, but with no intergroup difference (Table 2).
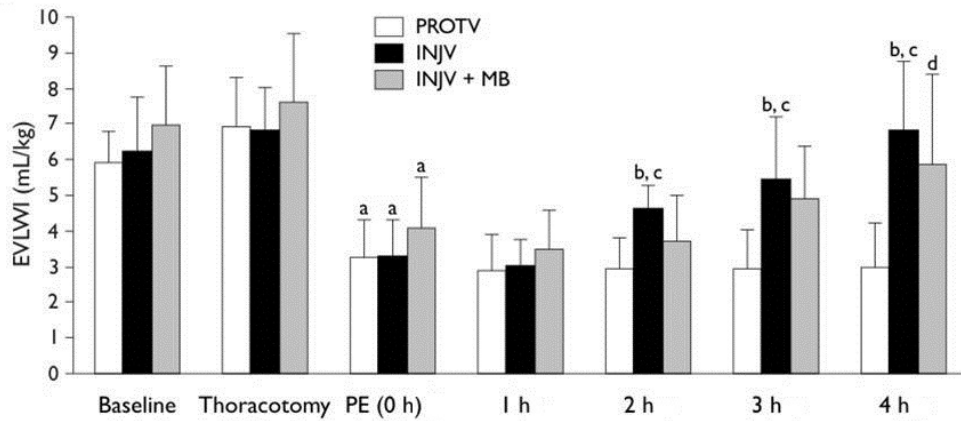
Figure 2. Extravascular lung water index (EVLWI) in sheep subjected to right-sided pneumonectomy followed by injurious ventilation. Methylene blue (MB) was administered from 1 hour and throughout.
PROTV, protectively ventilated group (n = 7); INJV, injuriously ventilated group (n = 7); INJV+MB, injuriously ventilated group treated with intravenous infusion of methylene blue (MB; n = 7).
Data are presented as mean ± SD. [a] $P < 0.05$ vs. baseline within group; [b] $P < 0.05$ vs. PE (0 h) within group; [c] $P < 0.05$ PROTV vs. INJV group; [d] $P < 0.05$ PROTV vs. INJV+MB group.

Table 2. Changes in blood gases, NOx and lung mechanics in sheep subjected to right-sided pneumonectomy followed by injurious ventilation. Methylene blue (MB) was administered from 1 hour and throughout.

	Groups	Baseline	Thoracotomy	PE (0 h)	1 h	2 h	3 h	4 h
pH	PROTV	7.50±0.05	7.56±0.08	7.52±0.07	7.52±0.07	7.53±0.07	7.52±0.08	7.51±0.08
	INJV	7.43±0.10	7.41±0.10[c]	7.40±0.09[c]	7.43±0.09[c]	7.41±0.08[c]	7.38±0.10[c]	7.34±0.14
	INJV+MB	7.44±0.04	7.44±0.03[d]	7.43±0.03[d]	7.43±0.05[d]	7.38±0.08[d]	7.36±0.10[d]	7.33±0.16[d]
PaCO$_2$, mm Hg	PROTV	36.0±3.8	29.3±6.0[a]	30.8±6.8[a]	31.5±6.0	31.5±6.0	31.5±5.3	33.0±5.3
	INJV	32.3±6.8	32.3±5.3	36.7±4.5	32.3±3.0	32.3±4.5	38.3±6.8	40.5±9.0
	INJV+MB	32.3±4.5	33.4±3.8	35.3±3.8	33.0±3.0	39.8±9.8	42.0±13.5	50.3±27.0
PaO$_2$/FiO$_2$, mm Hg	PROTV	408±84	383±83	334±58	380±76[b]	402±62[b]	400±64[b]	399±71
	INJV	390±42	345±69	301±130	407±81	345±83	236±150	186±131[a,c]
	INJV+MB	441±48	398±20	401±112	434±74	379±71	350±118	327±148
NO$_2$/NO$_3$, mM/L	PROTV	0.022±0.006						0.043±0.018[a]
	INJV	0.024±0.008						0.054±0.026[a]
	INJV+MB	0.020±0.008						0.041±0.011[a]
P$_{peak}$ cm H$_2$O	PROTV	19.0±3.6	15.9±3.0	35.5±7.3	33.0±6.50[b]	33.0±6.5	34.1±6.7	33.7±7.3
	INJV	17.5±2.7	14.0±4.1	32.4±5.6	40.3±5.9[b,c]	40.6±3.8[b,c]	44.6±4.9[b,c]	47.8±4.3[b,c]
	INJV+MB	16.4±2.9	15.8±4.3	32.0±4.8	41.7±6.1[b,d]	42.3±6.4[b,d]	44.8±5.8[b,d]	46.9±5.9[b,d]
P$_{plateau}$, cm H$_2$O	PROTV	16±4	13±3[a]	28±5[a]	26±3[a,b]	26±5[a]	27±5[a]	26±7[a]
	INJV	15±2	11±3[a]	27±3[a]	36±7[a,b,c]	36±4[a,b,c]	39±5[a,b,c]	44±5[a,b,c]
	INJV+MB	15±3	13±3	25±3[a,b]	36±6[a,b,d]	37±8[a,b,d]	39±6[a,b,d]	41±6[a,b,d]
C$_{qs}$, ml/cm H$_2$O/kg	PROTV	0.46±0.11	0.60±0.14	0.24±0.04[a]	0.26±0.03[b]	0.25±0.04	0.24±0.04	0.26±0.07
	INJV	0.42±0.06	0.58±0.12	0.24±0.03[a]	0.35±0.06[b,c]	0.34±0.04[b,c]	0.31±0.04[b,c]	0.28±0.04[b]
	INJV+MB	0.41±0.08	0.48±0.12	0.25±0.04[a]	0.34± 0.05[b,d]	0.33±0.07[b,d]	0.32±0.05[b,d]	0.30±0.06

PROTV, protective ventilated group (n = 7); INJV, injurious ventilated group (n = 7); INJV+MB, injurious ventilated group treated with intravenous infusion of methylene blue (n = 7).
Data presented as mean ± SD; h, hour; PaCO$_2$, arterial carbon dioxide tension; PaO$_2$/FiO$_2$, oxygen ratio;NO$_2$/NO$_3$, nitrite/nitrate; P$_{peak}$, peak airway pressure; P$_{plateau}$, airway plateau pressure; C$_{QS}$, total lung and chest quasistatic compliance.
[a] $P < 0.05$ vs. baseline within group; [b] $P < 0.05$ vs. PE (0 h) within group; [c] $P < 0.05$ PROTV vs. INJV group; [d] $P < 0.05$ PROTV vs. INJV+MB group

8.3. PAPER III: INHIBITOR OF NEURONAL NITRIC OXIDE SYNTHASE IMPROVES GAS EXCHANGE IN OVINE VENTILATOR-INDUCED LUNG INJURY AFTER PNEUMONECTOMY

Our objectives were to investigate whether the nNOS inhibitor 7-nitroindazole (NI) counteracts post-pneumonectomy pulmonary edema in sheep.

All the sheep survived without signs of aspiration, barotrauma or severe blood loss. We found no significant volumetric or hemodynamic differences between the groups at BL or PE. As shown in Figure 3, EVLWI increased by 88 % and 177 % in the INJV+ NI group and the INJV group, respectively (P<0.05). Correspondingly, PVPI increased by 100 % and 250 % (P<0.05), respectively (Table 3), but neither EVLWI nor PVPI differed significantly between treated and non-treated animals.

We found no significant changes in PBVI, whereas GEDVI and CI increased transiently in both the injuriously ventilated groups (P<0.05; Table 3). PAP and PAOP also increased in the injuriously ventilated groups, as compared to PE and to the PROTV group (P<0.05). At cessation of the experiments, PAOP decreased significantly in NI-treated sheep as compared to non-treated injuriously ventilated animals (P<0.05). In the PROTV and the INJV groups, SVRI declined after PE (P<0.05), but NI treatment prevented this decrease and increased PVRI as compared to the PROTV group (P<0.05; Table 3).

As shown in Figure 4, PaO_2 and SaO_2 increased towards the end of experiment in the INJV+NI group (P<0.05), and venous admixture increased in the INJV group (P<0.05). $PaCO_2$ increased and pH decreased in the INJV group, in comparison with PE and with the other groups (P<0.05; Table 4). After PE, SvO_2 increased transiently in all the sheep whereas Hb increased in the INJV group in comparison with both the intra- and the between group values (P<0.05; Table 4).

In the injuriously ventilated sheep, Ppeak increased in parallel with a decrease in C_{QS} (Table 4). The changes in Ppeak had similar patterns in both injuriously ventilated groups and differed both from their respective intragroup values at PE and from the PROTV group (P<0.05). Although not statistically significant, C_{QS} tended to be higher in NI-treated animals (Table 4).

The plasma concentration of NOx tended to increase throughout the experiment, but with no significant differences within or between the groups (Figure 5).

Total lung injury score was significantly higher in the INJV+NI group in comparison with the PROTV group (P<0.05; Table 5). As the individual data are concerned, hyaline membrane formation was more extensive in the INJV group as compared to the PROTV group (P<0.05) and significantly more neutrophils were found in NI-treated as compared to the non-treated INJV group (P<0.05). We found a trend towards a higher percentage of atelectases in the INJV group in comparison with the NI-treated group (28 % vs. 7%; not significant). Figure 6 shows photomicrographs of typical lung sections with low power fields (left). The marked squares are displayed as high power fields to the right. In the PROTV group (A), we found no pathological changes. The INJV group (B) displays hyperemia accompanied by extensive atelectases (atl), interstitial edematous thickening of the interalveolar and interlobular septa (ias and ils, respectively) - and of the subpleural interstitium (spi), and moreover, hyaline membrane (hm) formation and extravasation of neutrophils (arrows). The INJV+NI group (C), displays nearly the same changes with less atelectases (C).
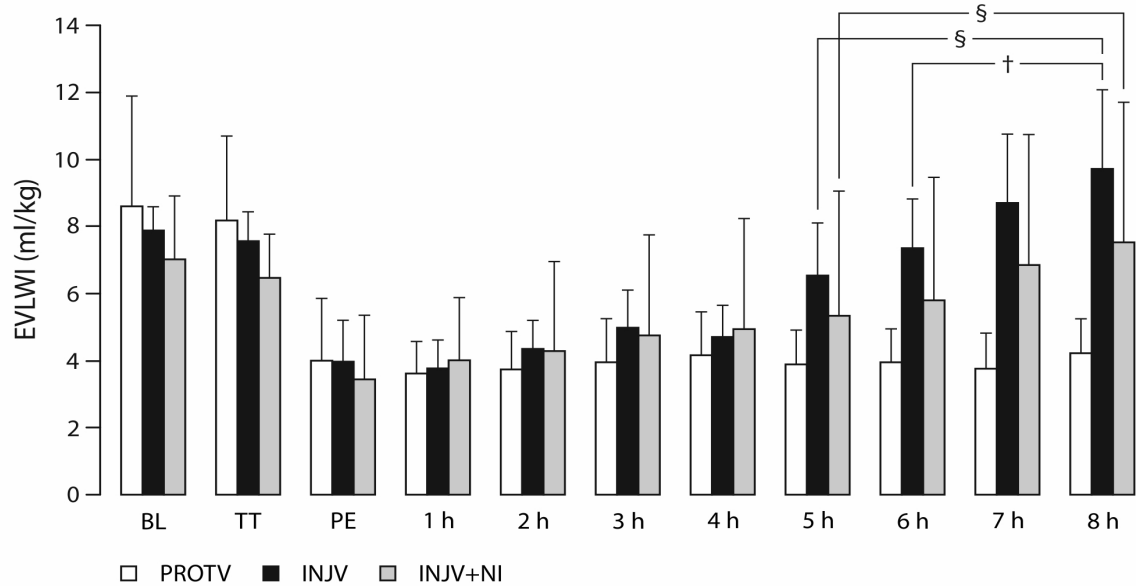
Figure 3 Extravascular lung water index (EVLWI) in anesthetized sheep. Baseline (BL), after right-sided thora-cotomy (TT) and after pneumonectomy (PE=time 0 hour) subsequently followed by injurious ventilation. 2 h – 8 h are time points in hours after PE. 7-nitroindazole (NI) was administered from time 2 h and throughout. Protectively ventilated group (PROTV; n = 8), injuriously ventilated group (INJV; n = 8), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 8). Data are presented as the mean ± standard deviation. § $P < 0.05$ within group in comparison with PE; † $P < 0.05$ between PROTV and INJV group.
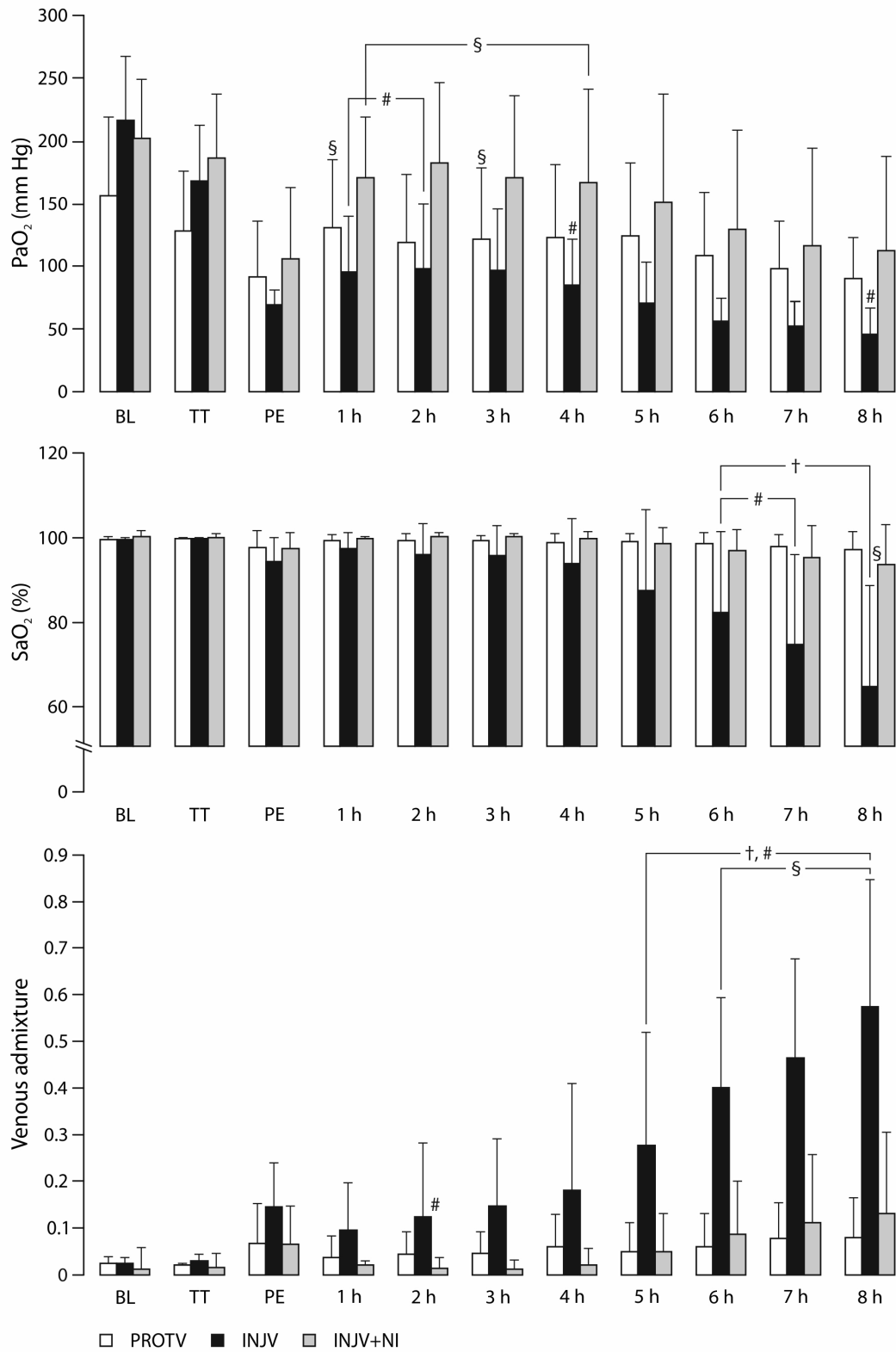
Figure 4. Arterial oxygen partial pressure (PaO$_2$), saturation (SaO$_2$) and venous admixture in anesthetized sheep. Baseline (BL), after right-sided thoracotomy (TT) and after pneumonectomy (PE=time, 0 hour) subsequently followed by injurious ventilation. 2 h – 8 h are time points in hours after PE. 7-nitroindazole (NI) was administered from time 2 h and throughout. Protectively ventilated group (PROTV; n = 8), injuriously ventilated group (INJV; n = 8), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 8). Data presented as the mean ± standard deviation. § P<0.05 within group in comparison with PE; † P < 0.05 between PROTV and INJV, # P < 0.05 between INJV and INJV+NI groups.
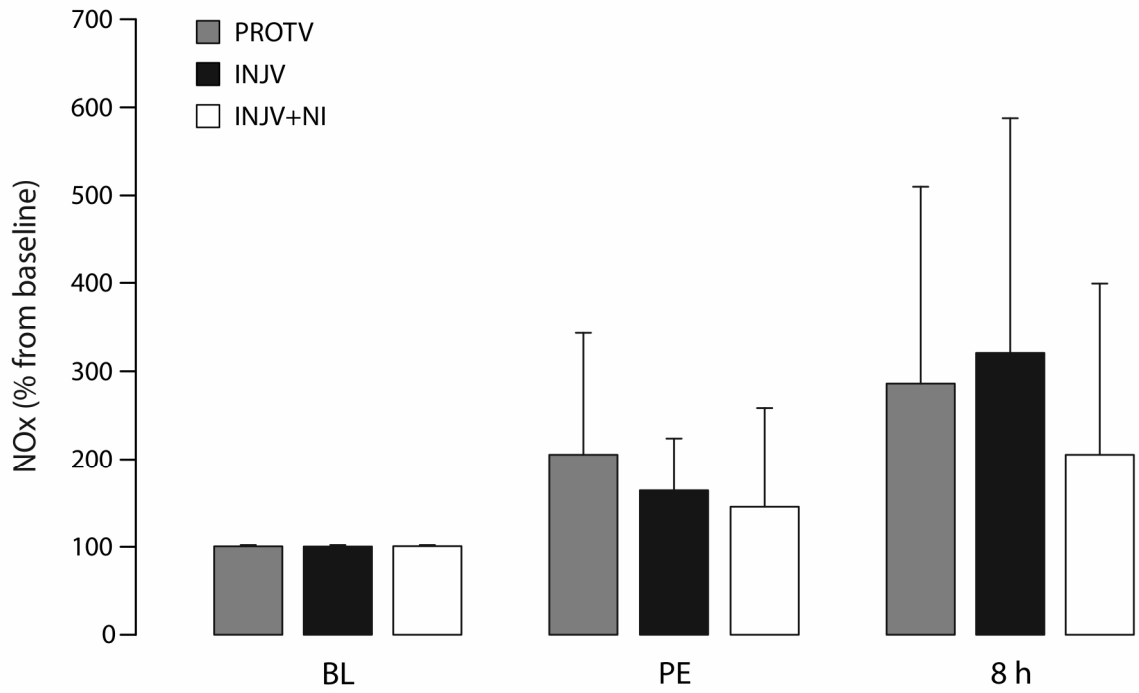
Figure 5. Changes in plasma concentrations of NOx in anesthetized sheep. Baseline (BL), after pneumonectomy (PE) subsequently followed by injurious ventilation and after euthanasia at 8 hours (8 h). 7-nitroindazole (NI) was administered from time 2 h (hours) and throughout. Protectively ventilated group (PROTV; n = 8), injuriously ventilated group (INJV; n = 8), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 8). Data presented as the mean ± standard deviation.
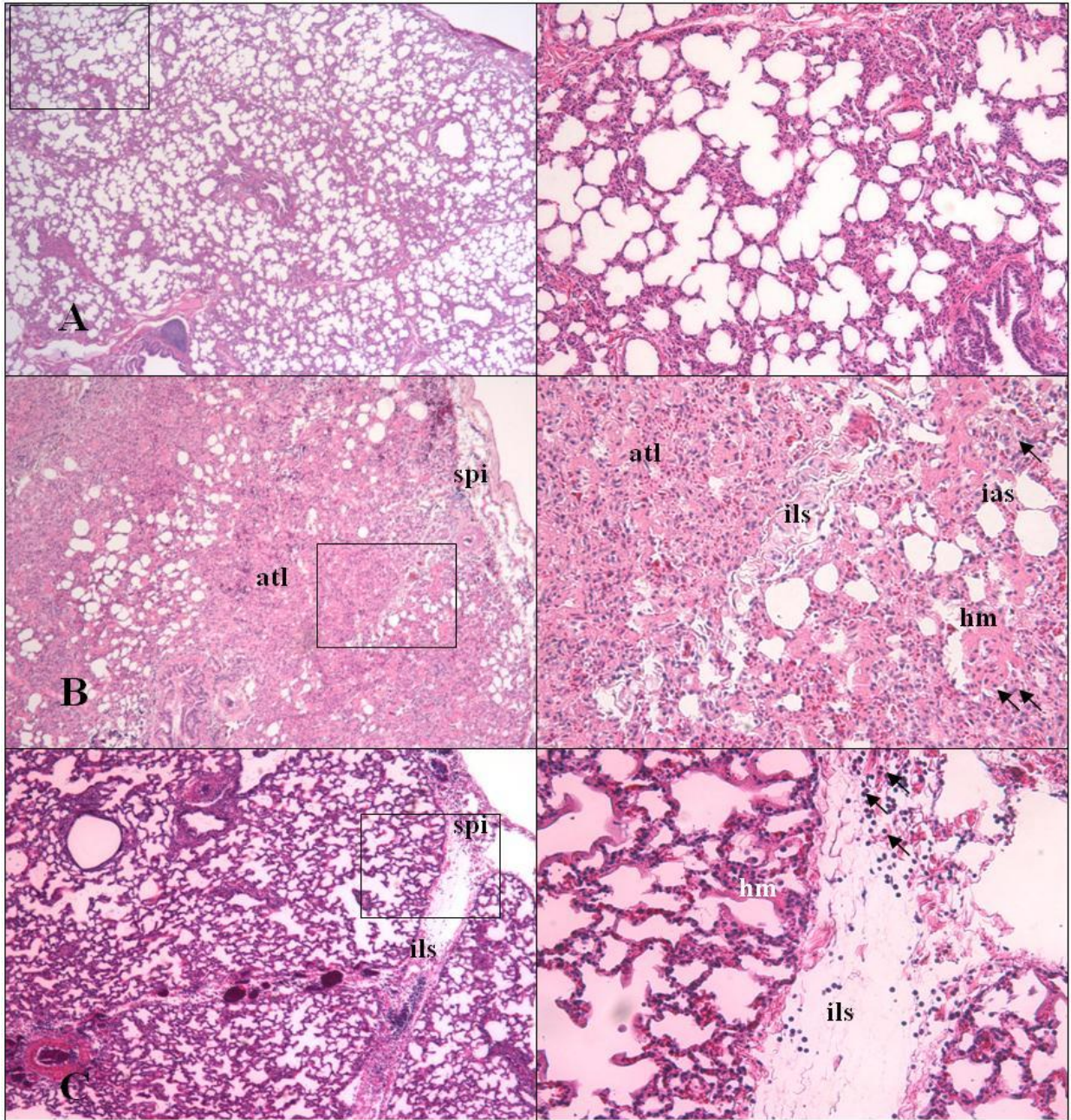
Figure 6. Lung histology. Photomicrographs of hematoxylin and eosin-stained left lung lower lobe specimens from sheep subjected to right pneumonectomy followed by an 8 h period of one-lung ventilation. Magnification x 25 (left). Areas within the squares are magnified x 100 (right).

Panel A: Protective ventilation (PROTV group) showing no pathologic changes.

Panel B: Injurious ventilation (INJV group)

Panel C: Injurious ventilation followed by intravenous infusion of 7-nitroindazole (INJV+NI group).

Atelectases (atl), interstitial edema with thickening of interalveolar and interlobular septa (ias and ils), thickening of the subpleural interstitium (spi), extravasation of neutrophils (arrows), formation of hyaline membranes (hm).

Table 3. Hemodynamic and volumetric variables of sheep subjected to pneumonectomy and ventilator-induced lung injury

Variable	Group	BL	PE	2 h	4 h	6 h	8 h
PVPI	PROTV	0.03±0.01	0.02±0.00	0.02±0.00	0.02±0.01	0.02±0.01	0.02±0.01[†]
	INJV	0.04±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.04±0.02	0.07±0.03[§]
	INJV+NI	0.04±0.02	0.03±0.02	0.03±0.03	0.03±0.03	0.04±0.05[§]	0.06±0.05[§]
PBVI (mL/m^2)	PROTV	285±127	196±74	210±75	215±62	206±69	210±65
	INJV	229±38	183±49	179±54	208±31	181±41	162±47
	INJV+NI	208±42	163±55	171±30	173±25	181±47	162±38
GEDVI (mL/m^2)	PROTV	602±127	557±175	537±138	539±144	567±124	612±144
	INJV	603±72	532±70	527±78	573±65	598±111[§]	622±160[§]
	INJV+NI	615±80	540±48	594±118	553±66	617±50[§]	590±65[§]
CI (L/min/m^2)	PROTV	3.4±0.8	3.9±1.9	4.5±1.7	4.4±1.7	4.7±1.4	4.6±1.6
	INJV	3.6±0.5	3.7±0.7	4.4±1.1	5.2±1.2[§]	5.2±1.3[§]	4.9±1.7
	INJV+NI	4.1±0.7	2.9±0.5	3.8±0.6[§]	3.7±0.6[§]	3.9±0.6[§]	3.5±0.6
PAP (mm Hg)	PROTV	12±2	19±5	20±3	21±4	20±4	22±7
	INJV	12±3	18±5	21±4[§]	25±5[§]	28±6[§,†]	31±6[§,†]
	INJV+NI	12±2	17±2	20±4[§]	23±7[§]	26±6[§]	28±6[§]
PAOP (mm Hg)	PROTV	6±3	6±3	6±3	9±4[§]	9±4[§]	8±4[§]
	INJV	5±2	8±3	8±3	12±4[§]	14±5[§,†]	16±4[§,†,#]
	INJV+NI	6±2	8±3	10±3[§]	9±4	11±4[§]	10±3[§]
SVRI (dyn sec/cm^{-5}/m^2)	PROTV	2096±641	2757±878	2001±749[§]	2039±923[§]	1851±877[§]	1889±872[§]
	INJV	2278±418	2389±572	2021±592	1682±491[§]	1547±480[§]	1717±514[§]
	INJV+NI	2034±371	2743±571	2352±647	2198±562	2299±432	2332±538
PVRI (dyn sec/cm^{-5}/m^2)	PROTV	129±53	286±104	258±123	247±146	220±81[§]	253±136
	INJV	164±73	231±34	238±54	212±51	233±78	276±125
	INJV+NI	128±56	266±103	242±72	299±130	333±155	426±122[‡]

Pulmonary vascular permeability index (PVPI), cardiac index (CI), pulmonary blood volume index (PBVI), global end-diastolic volume index (GEDVI), pulmonary artery pressure (PAP), pulmonary artery occlusion pressure (PAOP), systemic vascular resistance index (SVRI) pulmonary vascular resistance index (PVRI). Baseline (BL), thoracotomy (TT), pneumonectomy (PE), 2 h – 8 h are time points in hours after PE. Data are presented as the mean ± standard deviation. Protectively ventilated group (PROTV; n = 8), injuriously ventilated group (INJV; n = 8), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 8). [§] P<0.05 within group in comparison with PE; [†] P<0.05 between PROTV and INJV groups; [‡] P<0.05 between INJV+NI and PROTV groups; [#] P<0.05 between INJV+NI and INJV groups.

Table 4. Ventilation, blood gases and metabolic parameters of sheep subjected to pneumonectomy and ventilator-induced lung injury

Variable	Group	BL	PE	2 h	4 h	6 h	8 h
PaCO$_2$ (mm Hg)	PROTV	41.9±5.3	34.5±2.9	37.2±4.2	36.4±3.5	37.4±3.9	38.6±6.0
	INJV	44.2±3.4	42.1±7.1[†]	39.2±6.7	45.1±20.9	55.1±24.9	65.8±30.4[§,†,#]
	INJV+NI	40.6±3.2	36.2±4.4	32.5±5.2	35.8±4.2	39.8±6.7	37.1±3.8
pH	PROTV	7.44±0.05	7.50±0.05	7.49±0.05	7.49±0.06	7.48±0.07	7.45±0.11
	INJV	7.44±0.05	7.44±0.07	7.45±0.08	7.42±0.13	7.35±0.13[§,†]	7.28±0.17[§,†,#]
	INJV+NI	7.43±0.03	7.49±0.06	7.51±0.05	7.46±0.05	7.44±0.07	7.45±0.06
SvO$_2$ (%)	PROTV	71±9	58±13	72±11[§]	70±9[§]	68±6	62±11
	INJV	78±4	62±9	73±9[§]	74±10[§]	62±22	46±25
	INJV+NI	77±7	55±6	72±14[§]	74±12[§]	70±10[§]	59±16
Hb (g/L)	PROTV	87±6	87±5	94±13	92±14	91±13	85±12
	INJV	88±5	92±6	103±11[§]	109±13[§,†]	115±10[§,†,#]	124±15[§,†,#]
	INJV+NI	81±5	84±9	89±7	92±8	94±11	96±10
Ppeak (cmH$_2$O)	PROTV	20±7	42±9	40±9[†,‡]	45±16[‡]	47±15[†,‡]	50±18[†,‡]
	INJV	17±3	36±4	46±6[§]	49±5[§]	57±11[§]	61±16[§]
	INJV+NI	20±4	43±9	55±12[§]	60±15[§]	68±18[§]	67±18[§]
C$_{QS}$ (mL/cmH$_2$O/kg)	PROTV	0.53±0.20	0.25±0.08	0.27±0.07[§]	0.26±0.07	0.25±0.06	0.24±0.05
	INJV	0.45±0.05	0.21±0.03	0.32±0.04[§]	0.30±0.05[§]	0.25±0.05[§]	0.23±0.06
	INJV+NI	0.40±0.09	0.26±0.07	0.35±0.08	0.33±0.10	0.28±0.07	0.27±0.08

Partial pressure of carbon dioxide in arterial blood (PaCO$_2$), mixed venous oxygen saturation (SvO$_2$), hemoglobin concentration (Hb), peak airway pressure (Ppeak), quasi static compliance (C$_{QS}$). Baseline (BL), thoracotomy (TT), pneumonectomy (PE), 2 h – 8 h are time points in hours after PE. Data are presented as the mean ± standard deviation. Protectively ventilated group (PROTV; n = 8), injuriously ventilated group (INJV; n = 8), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 8). [§] P<0.05 within group in comparison with the 0 hours; [†] P<0.05 between PROTV and INJV groups; [‡] P<0.05 between INJV+NI and PROTV groups; [#] P<0.05 between INJV+NI and INJV groups.

Table 5. Histological lung injury score of sheep subjected to pneumonectomy and ventilator-induced lung injury

Group	Edema	Neutrophil infiltration	Hemorrhage	Hyaline membranes	Epithelial desquamation	Σ
PROTV	1.0 (0.0 to 1.0)	1.0 (0.0 to 2.0)	1.0 (0.0 to 3.0)	0.0 (0.0 to 2.0)	0.0 (0.0 to 1.0)	4.0 (1.0 to 7.0)
INJV	1.0 (1.0 to 3.0)	1.0 (0.0 to 3.0)	1.0 (0.0 to 2.0)	2.0 (2.0 to 3.0)[†]	1.0 (0.0 to 2.0)	5.0 (4.0 to 12.0)
INJV+NI	2.5 (2.0 to 3.0)‡	3.0 (2.0 to 3.0)‡,[#]	2.5 (1.0 to 3.0)	1.0 (1.0 to 2.0)‡	2.5 (2.0 to 3.0)	11.0 (11.0 to 12.0)

Data presented as the median (minimum to maximum). Protectively ventilated group (PROTV; n = 5), injuriously ventilated group (INJV; n = 5), injuriously ventilated group treated with intravenously infused 7-nitroindazole (INJV+NI; n= 5).
‡ P<0.05 between INJV+NI and PROTV; # P<0.05 between INJV+NI and INJV; † P<0.05 between PROTV and INJV group.

9. GENERAL DISCUSSION

The present thesis has shown that a non-cardiogenic pulmonary edema occurs in sheep after removal of one lung followed by injurious ventilation. The postpneumonectomy period is characterized by increased extravascular lung water content combined with increased pulmonary vascular permeability and increased intrapulmonary shunting giving rise to arterial hypoxemia.

Inspirated by studies in small animals (Peevy KJ *et al.*, 1990; Tajima A *et al.*, 2008) that opened for the possibility that mechanical ventilation with excessive tidal volumes and zero positive end-expiratory pressure might induce increased activity of NOS, we tested the effects of the inhibitor of NOS and guanylate cyclase, methylthionine (methylene blue; MB), on postpneumonectomy pulmonary edema. However, MB which is a combined inhibitor of *c*NOS and *i*NOS, failed to antagonize the development of lung edema when administered as treatment.

Investigations on models of acute lung injury after smoke inhalation combined with third degree burns or instillation of live bacteria into the airways, showed that the inhibitor of neuronal nitric oxide synthase (*n*NOS), 7-nitroindazole (NI), attenuated the derangement of gas exchange in parallel with improvement of lung morphology (Enkhbaatar P *et al.*, 2003). Hoping that NI would antagonize PPE, we started the administration of NI two hours after the commencement of injurious ventilation. We found that NI improves gas exchange, but failed to reduce lung water extravasation. The improvement of oxygenation and ventilation was interpreted as a result of restoration of hypoxic pulmonary vasoconstriction and normalization of the ventilation/perfusion relationship. Contradictory to these changes, histological lung injury score was not significantly reduced in the NI-treated animals.

9.1. SOCIO-ECONOMICAL AND ETHICAL CONSIDERATIONS

Postpneumonectomy pulmonary edema occurs with an incidence of 2 to 4% (Mathru M *et al.*, 1990; Turnage WS *et al.*, 1993) and leads to a mortality of 75-100% (Turnage WS *et al.*, 1993) without any significant decrease during the last decade. Such a high mortality rate might be explained by a suboptimal course of the surgical procedure and the perioperative period as well as by a lack of criteria for early detection of PPE (van der Werff YD *et al.*, 1997; Jordan S *et al.*, 2000; Algar FJ *et al.*, 2003). Those who survive PPE have longer ICU and hospital stays because the condition is not only limited to the lungs, but often complicates with MODS. In addition, treatment of PPE including a long stay in the ICU is an economic burden for the hospitals. Thus, PPE represents both a significant health care - and a socio-economical problem.

The data presented in this thesis were based on experiments on 75 yearling sheep that were euthanized at the cessation of the experiments. The use of large animals in the research of human medical conditions, such as pneumonectomy, raises ethical concerns encompassing features of the experimental model, the goals of research and their relationship with human health. Most of these issues are influenced by the ethical norms of the society. In most developed countries, human life is considered to be superior to that of animals. Therefore, most societies accept to sacrifice animals in order to improve prophylaxis and treatment of critical illness.

Norway has adopted the rules and regulations of the Federation of European Laboratory Animal Science Associations (FELASA) administered by the Norwegian Animal Research Authority. That document discusses the suffering of the animals against the potential benefits of the research for human health. However, it is puzzling to adequately assess the suffering of a sheep in the pre- and postoperative period. In the present study, the latter concern was non-existing since general anesthesia was induced and maintained throughout the surgical preparation and the sub-

sequent experiment, whereupon the sheep were euthanized. Cessation of life by euthanasia is often asked for by patients suffering unbearable pain from incurable cancer, although the procedure is considered to be unethical and abandoned in most countries.

Finally, euthanasia is an ethical issue which is influenced by culture, politics and religion. Most people consider human life as superior to that of animals and we have a tradition of utilizing animals both for food and clothing purposes. Therefore, it is also close at mind to use animals to enhance the knowledge of the pathophysiology and the treatment of life-threatening conditions like PPE. Experimental research with the ultimate goal to decrease postoperative mortality from PPE should be a strong motivation, which justifies the animal sacrifice.

It could be criticized that we used muscle relaxants in the experiments presented in Paper I, but not in those of papers II and III. However, in all the experiments, papers II and III alike, we paid strict attention to the responses of the sheep to avoid awareness and reactions from the sympathetic nervous system, such as changes in heart rate and blood pressure.

9.2. POSTPNEUMONECTOMY PULMONARY EDEMA

Pneumonectomy plays an important role among the risk factors for the development of ALI/ARDS, with additional perioperative factors including excessive intravascular volume resuscitation, extent of tissue resection and duration of operation, increased blood loss and reoperation (Zeldin RA *et al.*, 1984; Van der Werff YD *et al.*, 1997; Kutlu CA *et al.*, 2000; Martin J *et al.*, 2001). Investigators also have noticed that intraoperative tidal volumes used by some anesthesiologists during one-lung ventilation can exceed 10-12 ml/kg, which might put the patient at increased risk of developing lung edema (Jordan S *et al.*, 2000; Fuentes PA, 2003).

We noticed that removal of one lung followed by ventilation with excessive tidal volumes and ZEEP caused lung injury, as evidenced by an increase in extravascular lung water content. Extravascular lung water is an important determinant of acute lung injury, which has been suggested to play a role as an independent predictor of the course of illness and the prognosis (Bock J *et al.*, 1990; Mitchell JP *et al.*, 1992; Sakka SG *et al.*, 2002). Importantly, during the emergence of pulmonary edema, accumulation of EVLW occurs before any significant changes in blood gases, chest X-ray, or pulmonary vascular pressures. All the latter variables are nonspecific and influenced by a variety of factors (Pfeiffer UJ *et al.*, 1990; Bock J *et al.*, 1990; Boldt J, 2002). Thus, measurements of EVLW can probably help with early diagnosis and monitoring of PPE, since clinical signs such as dyspnea, hypoxemia, and markedly reduced lung compliance are unspecific and usually delayed by one to three days after surgery (Turnage WS *et al.*, 1993; Waller DA *et al.*, 1993).

EVLW and other volumetric variables presented in this thesis were measured by means of the double thermo–dye dilution (DTD) technique, as determined with COLD Z-021 (Pulsion Medical Systems, Munich, Germany). As shown in Paper I, these results agree closely with EVLW measured by *post mortem* gravimetry, which is considered to be the reference method of such measurements (Michard F, 2007). This issue was particularly focused on in a recent thesis from our group (Kuzkov V, 2006).

In both protectively and injuriously ventilated sheep, EVLW decreased immediately after lung removal (Papers I-III). Similar observations were reported after pneumonectomy in a pig model, where the authors observed a 27% decrease in EVLW (Roch A *et al.*, 2005). In contrast, by using an older version of the double-indicator technique, Lee and co-workers found that pneumonectomy does not increase the accumulation of EVLW in dogs (Lee E *et al.*, 1985). During the postpneumonectomy period, these animals were ventilated with room air at 12 breaths

per minute and a tidal volume of 8.5 mL/kg. The authors concluded that, provided the oncotic pressure is stable, the EVLW formation is related to the increase in left heart pressure, and this tendency is not enhanced by pneumonectomy. Moreover, the investigators argued that after removal of one lung, the lymphatics of the remaining lung are capable of keeping the interstitium clean (Lee E *et al.*, 1985).

The actual value of EVLWI also seems to depend on the pulmonary blood volume, which decreased after pneumonectomy in all the sheep, thus, explaining the initial reduction of EVLW (Papers I-III). After pneumonectomy, EVLWI remained stable in the protectively ventilated sheep, whereas a doubling of the tidal volume with application of ZEEP led to edema formation in the remaining lung. In healthy porcine lungs, Garcia-Delgado and co-workers failed to provoke significant lung injury by applying high tidal volumes over 4 hours, although histological signs indicated a possible initiation of lung injury (Garcia-Delgado M *et al.*, 2006). In contrast, in the present studies, PE followed by application of an aggressive ventilator setting induced a marked edema formation during the subsequent 4 hours, as shown in Papers I – II.

According to previous investigators, PPE is not a result of fluid overload or cardiac failure (Lichtwark-Aschoff M *et al.*, 1996). This statement was based on determination of intrathoracic blood volume index (ITBVI), which is considered to be a reliable marker of preload. Consistent with other recent investigations, this variable did not differ significantly between injuriously and protectively ventilated animals (Papers I-II; Luecke T *et al.*, 2004; Roch A *et al.*, 2005). It has been suggested that the emergence of lung edema either might be a result of increased pulmonary microvascular permeability or hydrostatic pressure, or a combination of both factors. The permeability changes might be prompted by a direct injury to the alveolocapillary membrane or arise as a consequence of an inflammatory process secondary to the surgical trauma as observed after pneumonectomy in patients (Mathru M *et al.*, 1990; Jordan S *et al.*, 2000). The latter assumptions are supported by findings reported in Paper III showing that pneumonectomy combined with injurious ventilation leads to loss of endothelial integrity and intrusion of edema fluid into the alveolar spaces.

Increased blood flow and volume in the remaining lung, in parallel with increased PAP and PAOP, are consequences of the significantly reduced volume of the pulmonary vasculature after the pneumonectomy. The increments in PAP and PAOP in the injuriously ventilated sheep might have elevated the pulmonary microvascular pressure albeit the effects on the fluid filtration pressure might have been partly outweighed by the increases in airway pressure secondary to the lung hyperinflation (Papers I-III). On the other hand, in dogs, investigators demonstrated that elevation of left atrial pressure to 25 mmHg by inflating a balloon in the left atrium caused an increase in EVLW independent of whether it was determined before or after PE. The authors concluded that pneumonectomy does not acutely increase the susceptibility to EVLW formation caused by a hemodynamic challenge. This implies that following pneumonectomy, if left heart filling pressures are normal, there is no clinical benefit from excessive fluid restriction (Lee E *et al.*, 1985). Since both the pulmonary venous and the arterial pressures may contribute to the increase in pulmonary microvascular pressure, it can be expected that any factor that increases PAP can lead to pulmonary edema. Increments in PAP after PE may be caused by active pulmonary vasoconstriction secondary to hypoxemia, hypercarbia or pain. It might also arise passively throughout the postpneumonectomy period due to increased cardiac output or left atrial pressure. Investigators also have noticed that PAP increases after pneumonectomy or bilobectomy, but remains unchanged in patients undergoing a lobectomy or resection of a segment (Kellow NH *et al.*, 1994).

In sheep that were subjected to aggressive ventilator settings, the postpneumonectomy period was associated with volumotrauma, which is consistent with the findings of previous workers (Dreyfuss D *et al.*, 1985; Tsuno K *et al.*, 1991; Parker JC *et al.*, 1993). In dogs, experimental overinflation of the lungs resulted in pulmonary edema (Albert RK *et al.*, 1980). In patients undergoing one-lung ventilation in whom the dependent lung is exposed to a relatively high tidal volume (8–10 mL/kg recommended), a high inspiratory driving pressure is often required to obtain adequate gas exchange. Application of a high intraoperative airway pressure has been identified as a special risk factor for ALI/ARDS (Van der Werff YD *et al.*, 1997). Szegedi et al. have noticed that institution of one-lung ventilation immediately increases the airway peak and plateau pressures. The latter notion agrees with the findings in Papers I-III (Szegedi LL *et al.*, 1997). Dreyfuss et al. demonstrated in rats that ventilation with tidal volumes up to 40 mL/kg in the presence of low transpulmonary pressure is more damaging to the lungs than ventilation with high airway pressures and normal tidal volumes (Dreyfuss D *et al.*, 1988). While experimental studies have demonstrated that small tidal volumes may result in a significant decrease in alveolar inflammatory cytokines (Tremblay L *et al.*, 1997), no differences were observed in the cytokine plasma levels of patients who randomly received either large or low tidal volumes during elective thoracic surgery (Wrigge H *et al.*, 2004). In contrast, Michelet and co-authors observed a significant increase in systemic inflammatory responses in patients during one-lung ventilation with large tidal volumes (Michelet P *et al.*, 2006). Moreover, large tidal volume also was identified as a risk factor of respiratory failure in a retrospective study of 170 patients who underwent pneumonectomy (Fernandez-Perez ER *et al.*, 2006).

In Paper III we found increased neutrophil infiltration, edema and hyaline membrane formation in the remaining lung of the injuriously ventilated sheep. Recently, investigators noticed that in rats PE combined with tracheal instillation of LPS induced a more severe lung injury with marked neutrophil infiltration, hemorrhage and lung edema, as compared with those subjected to pneumonectomy alone (Tajima A *et al.*, 2008). In patients undergoing PE because of benign or malignant lung diseases, preoperative neutrophil activation was related to the effects of the underlying lung tumor and the increased linear velocity of blood postoperatively. The latter increase the tangential and shear stress forces caused by the physical injury to the endothelium and led to increased lung permeability in the postoperative period (Waller DA *et al.*, 1996). More than two decades ago, Rocker and co-workers observed that increased pulmonary microvascular permeability was related to release of neutrophil elastase in patients with lung injury after esophagectomy (Rocker GM *et al.*, 1988). However, these findings contradict observations in pigs claiming that neutrophils are not involved in VILI. The latter investigators speculate that the alveolar instability in VILI is largely a result of mechanical stress and not of neutrophil-released proteases damaging the lung parenchyma (Steinberg JM *et al.*, 2004).

Different workers have noticed that pneumonectomy might increase the pro-coagulant activity and predispose to thrombus formation and embolization of the contralateral pulmonary artery (Kalweit G *et al.*, 1996). Physical stress due to injurious ventilation may trigger a further increase in pro-coagulant activity. Activation of coagulation may also trigger the inflammation cascades (Grichnik KP *et al.*, 2004; Tajima A *et al.*, 2008). Thus, after PE and injurious ventilation, it could well be that the two stimuli acted together to increase lung microvascular permeability, thereby promoting an increase in EVLW. In Paper III the effect of inflammation is evident from the observation that the edema score as well as the total histological lung injury score tended to be higher in injuriously ventilated sheep, as compared to the protectively ventilated animals. In this respect, our findings are consistent with investigations demonstrating that injuri-

ous ventilation causes a biotrauma that is characterized by inflammation and increased pulmonary microvascular pressure and permeability (Richard JD *et al.*, 2003).

In mechanically ventilated sheep, earlier workers found a positive correlation between the degree of lung inflation and the microvascular pore radius. The investigators noticed that the restriction of solute diffusion was lost when the lungs were ventilated at high inflation pressures and volumes, resulting in a net movement of liquid into the alveoli. The authors postulated that as the lung epithelium is progressively stretched there is an opening up of water-filled channels between the alveolar cells (Egan EA *et al.*, 1976). Our observation of significantly increased hemoglobin concentration in sheep that were injuriously ventilated over 8 hours (Paper III) supports the idea of leaks from the vasculature into the interstitial spaces. These findings are also consistent with those of Mandava and co-workers, who described a systemic capillary leak syndrome within 6 hours of high-pressure mechanical ventilation in sheep (Mandava S *et al.*, 2003). The latter also noticed cardiovascular changes and a profound hemoconcentration developing within 6 hrs of the start of aggressive mechanical ventilation, along with a major decline in pulmonary compliance and deterioration of arterial blood gases. Thus, our finding (Paper III) of increased hemoconcentration after PE followed by injurious ventilation is consistent with that observed in the same species after injurious ventilation alone (Mandava S *et al.*, 2003).

In addition to the increased outward filtration, mechanical ventilation has been shown to impede thoracic lymph flow in anesthetized dogs and sheep (Blomqvist H *et al.*, 1991; Maybauer DM *et al.*, 2006). At high airway pressures mechanical ventilation lowers the transmural pressures of lung lymph vessels, thereby reducing their diameter and the ability of the lymphatics to drain the lungs. Mechanical forces applied during the removal of a lung could also play a role in the development of PPE (Zapol WM *et al.,* 1979; Turnage WS *et al.*, 1993). In concert with aggressive ventilatory settings, particularly right pneumonectomy might reduce the lymphatic pump capacity by up to 50% (Peters RM, 1989). These factors, combined with increased blood flow through the remaining lung could increase the hydraulic filtration conductivity beyond the limited lymphatic drainage capacity of the remaining lung. Notably, some investigators have reported a higher incidence of PPE following a right-sided pneumonectomy as compared to a left sided pneumonectomy (Zeldin RA *et al.*, 1984; Turnage WS *et al.*, 1993), whereas other workers have not been able to confirm this observation (Waller DA *et al.*, 1993; Hayes JP *et al.*, 1995).

Protective ventilation of the remaining lung after PE combined with FiO_2 of 0.5 was sufficient to maintain normal gas exchange, as shown in Papers I-III. These data are in agreement with those of Filaire and co-authors, who observed normal gas exchange after PE in pigs ventilated with VT 7 ml/kg and ZEEP (Filaire M *et al.*, 2007). In contrast, our sheep that were injuriously ventilated after PE demonstrated prominent changes in gas exchange, reaching the oxygenation criteria of patients with ARDS. The gradual fall in PaO_2 and the rises in $PaCO_2$ and venous admixture in the INJV group may be explained by more extensive disturbances in the ventilation/perfusion relationships in comparison with the PROTV group. However, despite the fact that we used a PEEP of 4 cm H_2O in the PROTV group, and blood gases remained more unchanged throughout the experiments, lung histology revealed a tendency towards more atelectases in the latter animals compared to those exposed to injurious ventilation, although the difference did not reach statistical significance (Paper III). We suspect that the low tidal volumes used in the protectively ventilated animals might have facilitated airway collapse, which was less extensively expressed in injuriously ventilated animals that were exposed to the recruitment effects of higher tidal volumes and inflation pressures. On the other hand, the gradually occurring derangement of gas exchange in the injuriously ventilated animals, make us suspect that these

lungs might have been subjected to more ventilatory stress and strain as compared to the protectively ventilated group. The latter assumption is also supported by the finding of a higher lung injury score in the injuriously ventilated animals presented in Paper III. In a porcine model of oleic acid - induced ALI, Carvalho et al. found that the reduction in PEEP consistently increased the extension of poorly - or non-ventilated lung areas (Carvalho A *et al.*, 2007). In contrast, investigators ventilating pigs with primary healthy lungs with tidal volumes of 50 ml/kg and ZEEP over periods of until 4 hours did not detect any biochemical changes in lung lavage fluids and no histological signs suggestive of VILI (Garcia-Delgado M *et al.*, 2006). Thus, in larger animals, PPE might occur when PE is followed by injurious mechanical ventilation consisting of excessive tidal volumes and zero PEEP whereas such ventilation alone is not a sufficient stimulus for eliciting VILI over a foreseeable period of time. In contrast, according to several investigations over nearly four decades, injurious ventilation alone may provoke VILI in smaller animals, like in rats (Webb HH *et al.*, 1974). Based on our own findings and those of previous investigators, we suggest that mechanical ventilation employing excessive tidal volumes and zero PEEP is a prerequisite for PPE and that VILI is an integral part of this life-threatening condition. Consequently, it was close at mind to focus on VILI when trying to find a cure against PPE. As of today, protective mechanical ventilation employing small tidal volumes in combination with PEEP titrated to an optimal level above the lower inflection point of the lung pressure – volume curve, or high frequency oscillatory ventilation, appears to be the first steps taken to assist ventilation in patients who are unable to breathe after a pneumonectomy. However, in cases of full-blown PPE, extracorporeal membrane oxygenation (ECMO) by means of a partial veno-arterial bypass appears to be the ultimate therapeutic alternative. By using ECMO, it is possible at the same time both to induce "lung rest" and to unload the lung circulation, simultaneously securing the oxygen supply.

9.3. IS THE L-ARGININE/NO PATHWAY INVOLVED IN THE PATHOGENESIS OF POSTPNEUMONECTOMY PULMONARY EDEMA?

The pathogenesis of PPE is still grossly unsettled. Since injurious ventilation and ZEEP seemed to be important prerequisites for the evolvement of PPE, while ventilation with small tidal volumes and PEEP protected against the condition, we assumed that pharmacological therapies that could be expected to act on VILI also could be of potential benefit against PPE. A few years ago, investigators demonstrated that ventilation of rats with high tidal volumes and low PEEP induced a lung injury in parallel with release of cytokines into the systemic circulation. The ensuing systemic inflammatory response syndrome reminded on that seen in ALI and ARDS and the authors suggested that the condition could have relevance for the development of multisystem organ failure (Chiumello D *et al.*, 1999). Held and co-workers confirmed that excessive ventilation evokes early inflammatory responses similar to those stimulated by lipopolysaccharide (i.e., translocation of NF-kappa β and release of proinflammatory mediators), but according to these workers, the inflammatory responses were apparently independent of activation of Toll-like receptor 4 (TLR-4) (Held H *et al.*, 2001). In contrast, recent research on mice reveals that TLR-4 is quite pivotal for the initiation of innate immune responses to VILI (Vaneker M *et al.*, 2008, Vaneker M *et al.*, 2009). However, whether the chain of events taking place after injurious ventilation in mice also apply in large animals, like the sheep, has not been settled and will not be the subject of further discussion in this thesis.

As an integral part of the inflammatory response, nitric oxide generated from the amino acid L-arginine, may cause damage to cells and tissues by reacting with the superoxide anion to pro-

duce peroxynitrite, a highly reactive nitrogen species, which promotes lipid peroxidation (Liaudet L *et al.*, 2000). In experiments on isolated perfused rabbit lungs, Broccard and his co-workers noticed that the non-specific inhibitor of NOS, L-NAME, impaired the development of VILI, as judged by a lessening of the increase in microvascular permeability in parallel with a reduction of NO metabolites in bronchoalveolar lavage fluid (Broccard AF *et al.*, 2004). The latter findings inspired us to test the effects of another inhibitor of NOS, methylthionine (methylene blue; MB) on PPE in sheep.

Evidently, MB inhibits the generation of NO from both constitutive nitric oxide synthase (*c*NOS) and inducible NOS (*i*NOS). Additionally, it hampers the activation of guanylate cyclase and the formation of cyclic guanosine monophosphate and attenuates both vaso- and bronchodilation. MB also hampers cyclooxygenase-induced activation of arachidonic acid derivatives, as evidenced by inhibition of thromboxane-B2 and 6-keto-prostaglandin - $F_{1\alpha}$, and of the febrile response to endotoxin in sheep. Thus, we reasoned that MB could potentially reduce the generation of peroxynitrite from NO and superoxide, thereby counteracting the disintegration of lung microvasculature and the subsequent progress of lung injury, as we had experienced after infusion of LPS in sheep (Evgenov OV *et al.*, 2001; Evgenov OV *et al.*, 2002). However, we were surprised by the finding in Paper II that MB administered 1 h after the onset of injurious ventilation failed to attenuate PPE, as assessed by lack of changes in gas exchange and EVLW. Moreover, we noticed no significant difference in the plasma concentration of NO_2/NO_3 between MB-treated and non-treated animals. Since the lung injury did not improve, we reasoned that the duration and the dose of MB administration could be a limitation. The results contrast those obtained in previous investigations on endotoxemic sheep from our group. The latter showed that administration of MB was associated with reduced pulmonary capillary pressure and permeability-surface area product, improved gas exchange and a reduction of the increments in plasma nitrites and nitrates (Evgenov OV *et al.*, 2001; Evgenov OV *et al.*, 2001). As Paper II is regarded, we cannot exclude the possibility that both the sample size and the dose of MB were too small to display any pharmacological effects on hemodynamics, EVLWI and gas exchange. By using the data obtained in Paper II retrospectively to analyze sample sizes with a power of 80% and a significance level of 0.05, we found that approximately 15, 33 and 1000 animals in each group would be required to display significant effects of MB on PaO_2/FiO_2, PVPI and EVLWI, respectively (Altman DG, 1991).

Previous workers from our laboratory have demonstrated that a bolus injection of MB (10 mg/kg), as pretreatment, subsequently followed by an infusion of 2.5 mg/kg/hr over 5 hours, attenuates the deterioration of hemodynamics and gas exchange in ovine endotoxin-induced lung injury (Evgenov OV *et al.*, 2001). Although mere speculation, it is not unlikely that the drug effect would have been greater if MB had been administered from immediately after PE. In contrast to our findings, investigators studying interstitial ischemia-reperfusion injury in rats demonstrated that a 4-hr exposure to MB significantly reduced the wet-to-dry-weight ratio and the histological damage to the lungs (Galili Y *et al.*, 1998). Another point to consider is that we aimed at giving a dose that was more comparable with that we had used for patients with septic shock in an earlier investigation (Kirov MY *et al.*, 2001).

Thus, infusion of MB neither reduced the increased generation of NO - metabolites (NO_2/NO_3) nor the development of lung injury in this model of PPE. Therefore, further studies are warranted to determine whether other parts of the L-arginine/NO-system or other mediator systems could be involved in the evolution of pulmonary edema after PE in sheep.

Recently, investigators studying different models of ALI in various species, have suggested the possibility that neuronal nitric oxide synthase (nNOS) is involved in the pulmonary vascular responses to pathological insults (Fisher A et $al.$, 1996; DeSanctis GT et $al.$, 1997; Feletou M et $al.$, 2001). Lange and co-workers, who studied acute lung injury in response to smoke inhalation and burns over 48 h in sheep, found that the specific inhibitor of nNOS, 7-nitroindazole (NI), reduced the plasma concentration of NO_2/NO_3 and attenuated the increase in pulmonary shunt fraction most efficiently during the first 12 h. On the other hand, inhibition of iNOS proved more effective during the second 12 h of the experiments (Lange M et $al.$, 2009). These reports inspired us to hypothesize that given the fact that lung injuries of different etiologies seem to elicit the same immunological responses we should not exclude the possibility that an inhibitor of nNOS also might dampen PPE. Since the duration of exposure to the injurious stimulus as well as to the treatment could have been decisive for the lack of effect of MB, we planned the experiments of Paper III to last twice as long as those presented in Paper II. However, as reported in Paper III, the effect of NI on this model of PPE was grossly limited to an improvement of the decrease in gas exchange. These findings were consistent with recent investigations showing that NI improves gas exchange in other ovine models of lung injury such as after smoke inhalation and burns (Westphal D et $al.$, 2008) and smoke inhalation followed by instillation of live bacteria into the airways (Enkhbaatar P et $al.$, 2003). Despite NI slightly delayed the changes in EVLWI and pulmonary vascular permeability, lung tissue from NI-treated animals displayed no reduction of the histological lung injury. These findings contrast with those made by investigators studying ALI after inhalation of smoke and instillation of bacteria into the airways, or after third degree burns, who noticed that inhibition of nNOS improved lung mechanics and reduced the histological signs of airway obstruction (Enkhbaatar P et $al.$, 2003; Enkhbaatar P et $al.$, 2009).

It is close at mind to criticize that we have no indication of nNOS mRNA expression or protein formation in lung tissue of these animals. In another recent study the investigators found increased expression of eNOS after PE in rats (Samano MN et $al.$, 2009). Correspondingly, Peng and his co-workers observed up-regulation of iNOS in parallel with increased leakage from alveolar capillaries of mice subjected to injurious ventilation. Notably, specific inhibition of the activity of iNOS, by means of aminoguanidine, for the greater part prevented the lung injury. The investigators also observed that iNOS-deficient mice were protected against the injury to mechanical ventilation (Peng X et $al.$, 2005).

Several investigations have demonstrated that injurious ventilation causes a biotrauma, which is an inflammatory reaction that increases pulmonary capillary permeability (Hoegl S et $al.$, 2008). In Paper III, the fact that interstitial edema, neutrophil infiltration and hyaline membrane formation were more extensive in the injuriously ventilated groups, as compared to the PROTV group, indicates that inflammation is involved in this lung injury. We were surprised to find that the changes were slightly but not significantly more prominent in the NI-treated animals. It is worthy of note that a literature search revealed no previous studies focusing on inflammation and lung histological changes after combined pneumonectomy and injurious ventilation in sheep.

Endothelial integrity is lost in patients with PPE, as demonstrated by an increase in the protein content of broncho-alveolar lavage fluid (Mathru M et $al.$, 1990). Our assumption that vascular leaks occur in the extrapulmonary parts of the circulation was supported by the observation of increased Hb concentration in the INJV group, while lower Hb levels were found in NI-treated animals. Since EVLWI did not differ between the treated and the non-treated injuriously ventilated groups, this could be taken as an indication that NI reduced the leakage from tissues outside the lungs. Alternatively, the reduced hemoconcentration of the NI-treated sheep could result

from increased lymphatic drainage of the lungs. Such a mechanism was suggested by our group after the observation of increased lymphatic drainage of the lungs after the administration of the inhibitor of inducible nitric oxide synthase, aminoguanidine to sheep subjected to endotoxin-induced lung injury (Evgenov OV *et al.*, 2000). In contrast to our findings, workers studying smoke inhalation lung injury demonstrated reduced lung water extravasation after administration of the specific *n*NOS inhibitor ZK 234238 (Enkhbaatar P *et al.*, 2009).

Previous studies have shown that lung injury after endotoxemia, and systemic inflammation with excessively produced NO, and smoke inhalation alone - or in combination with burns impair HPV (Thiessen JL *et al.,* 1990; Fischer SR *et al.*, 1997; Ichinose F *et al.*, 2003; Westphal M *et al.*, 2006). Despite our inability to demonstrate significant changes in the plasma concentrations of NO_2/NO_3 (Paper III), we suggest that enhanced local production of NO might have contributed to local vasodilatation and consequently to dampening of HPV in poorly ventilated – or atelectatic lung areas, thereby causing increased intrapulmonary shunting and a decrease in arterial oxygenation in the INJV group. We believe that the improvement of gas exchange in the INJV+NI group was caused by reinforcement of HPV concerted by more favorable V/Q distribution following inhibition of NOS. However, our experiments were not designed to decide whether NI acted *via* selective inhibition of nNOS or non-specifically. Similar findings have been reported in previous studies of inhibitors of isomers of NOS in a variety of ovine models of lung injury (Evgenov OV *et al.*, 2000; Evgenov OV *et al.* 2001; Westphal M *et al.*, 2008; Lange M *et al.*, 2009).

In the experiments included in Paper III, the time of exposure to the drug could be a limitation of the study. The dose of NI used in our investigation was the same as used in other recent studies, where the authors found that a dose of 1 mg/kg/h was sufficient to reduce NOx plasma levels and to preserve HPV, without a further increase in HPV with higher doses (Lange M *et al.*, 2009). In previous studies of acute ovine lung injury, administration of the specific *n*NOS inhibitor was started one hour after the insult, and the experiments were performed awake over 24–48 hrs (Enkhbaatar P *et al.* 2003; Westphal M *et al.*, 2008; Enkhbaatar P *et al.* 2009). In contrast, in our experiments (Paper III), injurious ventilation lasted only 8 hrs and the administration of NI started two hours after the commencement of injurious ventilation. It is noteworthy, however, that after ovine smoke and burn lung injury, the authors reported the first sign of improved oxygenation after administration of NI between 6 and 12 hours after the injury, as assessed by the decrease in intrapulmonary shunt (Lange M *et al.*, 2009). This is consistent with the findings presented in Paper III, where a significant increase in venous admixture was observed in the INJV group from 5 h after PE and throughout the experiment as compared to the improvement displayed by the NI-treated animals.

We have no good explanation for the discrepancy between the improvements of gas exchange and the lung histological changes that did not improve during the exposure to NI. So, apparently, further studies are warranted to find a pharmacological compound which also antagonizes the lung injury per se.

10. PERSPECTIVES

Animal models play an important role in pathophysiological and pharmacological studies of ALI and ARDS. We have shown in sheep that following pneumonectomy, a change in lung ventilation from a protective – to an injurious mode with zero end-expiratory pressure, causes a significant derangement of arterial oxygenation and a concomitant rise in extravascular lung water. We believe that this model gives a unique possibility to study PPE in a large animal. In future studies the exposure to the injurious ventilation should be extended at least to 12 h in order to simulate more realistically the therapeutic challenges associated with these patients in the ICU. As the sheep genome will be more clarified, these projects might also be changed into a more translational course.

The fact that we failed to demonstrate any effects of MB on the lung injury following excessive ventilation after PE could be due both to the dose of MB and the time of exposure. It is not unlikely that the drug effect could have been greater if MB had been administered at a higher dose from immediately after PE and throughout an extended period of injurious ventilation. Since iNOS is more expressed after 12 h we could also have expected a dampening effect of MB beyond this time point (Lange M *et al.*, 2009).

It has been suggested that neuronal NOS (nNOS) is involved in the pathogenesis of ALI (Fisher A *et al.*, 1996; DeSanctis GT *et al.*, 1997; Feletou M *et al.*, 2001). In a sheep model of smoke inhalation and burns it was found that the specific inhibitor of nNOS, 7-nitroindazole, dampened the progression of lung injury (Lange M *et al.*, 2009). These findings were consistent with other investigations showing that 7-nitroindazole improves gas exchange in other models of ovine lung injury (Westphal D *et al.*, 2008; Enkhbaatar P *et al.*, 2003). In our model of PPE, 7-nitroindazole improved gas exchange, but did not reduce lung water extravasation. Therefore, further studies are warranted to determine whether other parts of the L-arginine/NO-system or other mediator systems could be involved in the evolution of pulmonary edema after PE in sheep.

In rats, hyperinflation of the remaining lung caused lung tissue damage with release of high mobility group box 1 protein (HMGB1) both from damaged cells and from activated neutrophils and macrophages (Wang H *et al.*, 1999). HMGB1 has been shown to play a role in the emergence of VILI in intact rabbit lungs (Ogawa EN et al., 2006) and after PE in mice (Tajima A *et al.*, 2008). In patients after thoracic esophagotomy, the postoperative plasma concentration of HMGB1 was higher in those who presented with a complicated postoperative course (Suda K *et al.*, 2006). Thus, the role of HMGB1 in the pathogenesis of PPE in large animals has not been settled and might become a target of future investigations.

Recent research has revealed that toll-like receptor 4 (TLR-4) is important for the initiation of innate immune responses to VILI in mice (Vaneker M *et al.*, 2008, Vaneker M *et al.*, 2009). However, whether these findings apply for large animals has not been settled, and could be a subject of further research.

It has been abundantly demonstrated that lung injury after endotoxemia, systemic inflammation with excessively produced NO, and smoke inhalation alone -, or in combination with burns, impairs HPV (Thiessen JL *et al.*, 1990; Fischer SR *et al.*, 1997; Ichinose F *et al.*, 2003; Westphal M *et al.*, 2006). This is in part thought to be due to the generation of ROS (Chabot F *et al.*, 1998; van der Vliet A *et al.*, 1999). Thus, an intriguing question could be whether inhibitors of ROS could be of potential benefit by antagonizing impaired vasoconstriction in response to alveolar

hypoxia thereby improving the outcome from PPE and other forms of VILI. All these research goals should be elucidated in experiments on large animals to uncover more of the pathophysiology and potential pharmacological approaches for future prophylaxis and treatment of PPE.

11. SUMMARY

Pneumonectomy in sheep followed by ventilation of the remaining lung with excessive tidal volumes and zero end-expiratory pressure induced a lung injury, characterized by derangement of gas exchange and pulmonary edema. By exposing the lungs to a protective ventilation mode consisting of tidal volumes of 6 ml/kg with positive end-expiratory pressure of 2 - 4 cm H_2O, gas exchange remained within physiological limits and no increase in extravascular lung water was noticed. This is a novel model of ventilator-induced lung injury in sheep.

In small animals, injurious ventilation employing high tidal volumes triggered the L-arginin/nitrogen monoxide system, and administration of unselective inhibitors, counteracted the ventilator-induced lung injury. Inspirited by these observations, we tested the hypothesis in excessively ventilated sheep that the non-selective inhibitor of NOS, methylene blue, prevents postpneumonectomy pulmonary edema. However, we observed no decrease in extravascular lung water or increase in arterial oxygenation that could support a beneficial effect of methylene blue. On the other hand, intravenous infusion of the inhibitor of neuronal nitrogen monoxide synthase (nNOS), 7-nitroindazole improved the gas exchange but failed to prevent an increase in extravascular lung water and histological signs of lung injury. Further studies are warranted to find out whether 7-nitroindazole counteracts ovine post-pneumonectomy pulmonary edema after exposure to a longer period of injurious ventilation.

12. CONCLUSIONS

1. Ventilator-induced lung injury of the remaining lung after pneumonectomy is a novel experimental model of lung injury in a large animal.
2. In injuriously ventilated sheep, the postpneumonectomy period was characterized by increments in extravascular lung water and pulmonary vascular permeability, and increased intrapulmonary shunting.
3. We suggest that ventilator-induced lung injury may act as a co-factor in combination with pneumonectomy to trigger the postpneumonectomy pulmonary edema.
4. The L-arginine/NO system does not seem to play a major role in the pathogenesis of PPE.
5. Infusion of MB, an inhibitor of *c*NOS and *i*NOS, neither prevented the increased generation of NO metabolites nor the emergence of lung injury.
6. Intravenous infusion of 7-nitroindazole, started two hours after the commencement of injurious ventilation, improved gas exchange but did not counteract lung injury with pulmonary edema and morphological injury in these experiments over eight hours.
7. Other experimental compounds should be investigated for potential involvement in the pathogenesis of PPE to decide whether its modulation could be suited as a target for the treatment of this subtype of ALI.

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Paper I

Paper II

Paper III