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Investigation of the Role of the NF-κB Pathway in Mediating the Effects of Hypercapnic Acidosis in Prolonged Systemic Sepsis and Ventilation Induced Acute Lung Injury

by

Maya Contreras

[MD, FCARCSI]

PhD Thesis

Supervisor: Professor John G Laffey

Faculty of Medicine,

National University of Ireland, Galway

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Declaration

The scientific work presented in the thesis is the author’s own work. The author has not obtained any other degree in NUIG or elsewhere based on this body of work. The extent of contribution of other members of the research group is acknowledged and described in detail as it follows:

Dr. Brendan Higgins main contribution was to teach how to perform animal surgery, harvesting, BAL cell counts, lung wet: dry ratio, histology. He also gave valuable insights to model development in the CLP and VILI series. Bacterial counts and some of the BAL cell counts in the CLP series were performed by Dr. Higgins.

Dr. Daniel O’Toole main contribution was to teach, discuss various molecular biological methods, such as ELISA, protein assays, Western blot, RT-PCR, techniques to generate recombinant AAV vector and to assist and supervise my measurements. He also helped to acquire basic cell culture techniques for cell works. Determination of IκBαSR-GFP transgene induction and endogenous IκB-α gene induction after injurious ventilation in the viral vector series was performed by Dr. O’Toole. Also, determination of total IκB and nuclear p65 using ELISA in Chapter 7 was performed by Dr. O’Toole.

Dr. James Devaney main contribution was to help to carry out the vector work. This body of work was extremely robust and laborious, and required large amount of transfected cells and repeated purification processes. His contribution to generate the viral products which was sufficient for transfection was significant. Determination of viral titres was supervised and predominantly performed by Dr. Devaney. Flow cytometric analysis of transfected, uninjured lung epithelial cells was performed by Dr. Devaney.

Dr. Gerard Curley main contribution was to assist in the rAAV6-IκBα-SR series. Dr. Curley actively participated in the re-piloting of VILI, vector instillations, to carry out injurious ventilation in pre-treated animals, and data collections.

Claire Masterson created and provided Figure 1-1 in the Introduction.
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Finally, I want to thank to my parents and to my brothers, Juan, Raúl, Carlos and Roberto for their patience, guidance and unconditional love. There are no words to express my gratitude and love for them.
Dedication

This work is dedicated to my parents, Judit and Juan.

Este trabajo está dedicado a mis padres, Judit y Juan, que hicieron todo lo posible - y muchas veces también lo imposible - para ayudarme a encontrar mi camino.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>AECC</td>
<td>American European Consensus Conference</td>
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<tr>
<td>ALI</td>
<td>Acute lung injury</td>
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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BE</td>
<td>Base excess</td>
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<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
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<tr>
<td>CASP</td>
<td>Colon ascendens stent peritonitis</td>
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<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<tr>
<td>CLD</td>
<td>Chronic lung disease</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COO-Hb</td>
<td>Carboxyhaemoglobin</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
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<tr>
<td>CREB</td>
<td>cAMP response-element binding protein</td>
</tr>
<tr>
<td>CV</td>
<td>Conventional ventilation</td>
</tr>
<tr>
<td>DAAO₂</td>
<td>Alveolar arterial oxygen difference</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified eagle medium</td>
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<tr>
<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
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<tr>
<td>EGRF</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELSO</td>
<td>Extracorporeal life support organization</td>
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<tr>
<td>ESICM</td>
<td>European Society of Intensive Care Medicine</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine seum</td>
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21
FiCO₂ Fractional inspired CO₂ concentration
FiO₂ Fractional inspired O₂ concentration
GFP Green fluorescence protein
H⁺ Hydrogen ion
HbO₂ Oxyhaemoglobin
HCA Hypercapnic acidosis
HCO₃⁻ Bicarbonate ion
HFOV High-frequency oscillatory ventilation
ICAM-1 Intercellular adhesion molecule 1
ICU Intensive care unit
IkB-α/β/ε Inhibitor of kappa B α/β/ε
IkBα-SR IkB-α super-repressor
IKKα/β/γ Inhibitor of kappa B kinase α/β/γ
IL Interleukin
IR Ischaemia-reperfusion
ITRs Inverted terminal repeats
i.v. Intravenous
LIS Lung injury score
LPS Lipopolysaccharide
MAP Mean arterial pressure
MCS Multiple cloning site
MMP Matrix metalloproteases
MOD Multiple organ dysfunction
MOF Multiple organ failure
MPO Myeloperoxidase
n Number
NaHCO₃ Sodium bicarbonate
NF-κB Nuclear factor kappa B
NO Nitric oxide
NOₓ NO metabolites
PAI-1 Plasminogen activator inhibitor 1
PBS Phosphate buffer saline
PEEP Positive expiratory pressure
pH Ph
PIP Peak inspiratory pressure
PaO₂ Arterial oxygen tension
paCO₂ Arterial CO₂ tension
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$P_{\text{peak}}$</td>
<td>Peak pressure</td>
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<tr>
<td>$P_{\text{plat}}$</td>
<td>Plateau pressure</td>
</tr>
<tr>
<td>PV</td>
<td>Pressure-volume</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant adeno-associated virus</td>
</tr>
<tr>
<td>RCTs</td>
<td>Randomized controlled trials</td>
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<tr>
<td>RDS</td>
<td>Respiratory distress syndrome</td>
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<td>RHD</td>
<td>N-terminal homology domain</td>
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<tr>
<td>RR</td>
<td>Respiratory rate</td>
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<tr>
<td>Rpm</td>
<td>Revolution per minute</td>
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<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>SCCM</td>
<td>Society of Critical Care Medicine</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SP-D</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>SSF</td>
<td>Sero-sanguineous fluid</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
</tr>
<tr>
<td>THAM</td>
<td>Tris-hydroxymethylaminomethane</td>
</tr>
<tr>
<td>sTNFr-1</td>
<td>Soluble TNF receptor 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>V/Q</td>
<td>Ventilation/Perfusion</td>
</tr>
<tr>
<td>VILI</td>
<td>Ventilation induced lung injury</td>
</tr>
<tr>
<td>vs.</td>
<td>Versus</td>
</tr>
<tr>
<td>$V_t$</td>
<td>Tidal volume</td>
</tr>
<tr>
<td>Vwf</td>
<td>Von Willebrand Factor</td>
</tr>
</tbody>
</table>
ABSTRACT

Background: Lung protective ventilation is integral to the management of ARDS. The resulting respiratory acidosis [HCA], termed “permissive hypercapnia”, is protective in diverse non-septic acute lung injury [ALI] models, but worsens bacterial pneumonia induced ALI. NF-κB is a key transcription factor regulating lung injury, inflammation, and repair. Our aim was to investigate the role of NF-κB pathway mediating the effects of HCA in in vivo septic and non-septic ALI models. We hypothesised that the effects of HCA are mediated by NF-κB inhibition, and that direct inhibition of NF-κB would exert similar protective effects.

Methods: Following model development pilot studies, four sets of experiments were performed. (1) Caecal ligation and puncture [CLP] was used to develop an in vivo prolonged sepsis induced ALI model. Sprague-Dawley rats were exposed to normocapnia or HCA for 96 hours after CLP. (2) Two in vivo ventilation induced lung injury [VILI] models with distinct injury severities were developed. Sprague-Dawley rats were exposed to normocapnia or HCA while were ventilated with either moderate or severe VILI protocols for 4 hours. (3) An IκB-α-super-repressor containing adeno-associated viral vector system [rAAV6-IκBα-SR] was constructed. Sprague-Dawley rats received rAAV6-IκBα-SR, viral vector without transgene or surfactant via intratracheal instillation. After 96 hours animals underwent VILI. In all studies, animal survival, physiologic and structural indices of lung injury severity, cytokine concentrations, and indices of activation of the NF-κB pathway [including transgene expression where relevant] were assessed.

Results: (1) Sustained HCA attenuated systemic sepsis induced ALI and reduced NF-κB activity. Importantly, HCA did not increase bacterial load in CLP sepsis. (2) HCA protected against VILI by inhibiting the NF-κB pathway. (3) Intrapulmonary delivery of AAV6-IκBα-SR conferred direct protection against VILI.

Conclusion: HCA protected against sepsis and ventilation induced ALI and this appears to have been mediated, at least in part, by NF-κB pathway inhibition.
CHAPTER 1
CHAPTER 1 : INTRODUCTION

1.1 ACUTE RESPIRATORY DISTRESS SYNDROME

1.1.1 DEFINITION AND DIAGNOSIS

1.1.1.1 Initial description

Acute respiratory distress syndrome [ARDS] was first reported by Ashbaugh and colleagues in 1967 during the Vietnam War [1]. The authors observed 12 patients whom presented with acute onset of severe dyspnoea, tachypnoea, oxygen refractory cyanosis, reduced lung compliance, and diffuse alveolar infiltration on chest radiograph. Since the patients displayed clinical symptoms remarkably similar to infantile respiratory distress syndrome, Petty and Ashbaugh defined the symptoms as adult respiratory distress syndrome [1971] to distinguish it from the respiratory distress syndrome of neonates [2]. The following two decades there were no standardized diagnostic criteria for ALI/ARDS and most of the definitions in studies were based on the initial clinical description by Ashbaugh.

1.1.1.2 Murray Score

In 1988, Murray et al developed a lung injury severity score [LIS]. The objectives of the LIS were to define whether the condition was acute or chronic; to assess the severity of the injury using a composite score derived from oxygenation, positive end expiratory pressure [PEEP], chest radiologic appearance, and respiratory compliance; and to determine what underlying pathologies caused or were associated with lung injury [3] [Table 1-1;Table 1-2]. The importance of the LIS score was to introduce a new concept and emphasize that ARDS manifests as a syndrome with variable severity rather than a single entity.

<table>
<thead>
<tr>
<th>Extended definition of ALI/ARDS proposed by Murray and colleagues [3]</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Course of the lung injury: acute or chronic</td>
</tr>
<tr>
<td>• Severity of physiological lung injury as determined by a composite lung injury score</td>
</tr>
<tr>
<td>• Lung injury caused by or associated with known risk factors for ARDS [direct or indirect lung injury]</td>
</tr>
</tbody>
</table>

Table 1-1 Extended three part definition of ALI/ARDS proposed by Murray et al including the course of lung injury; lung injury score [LIS] describing the severity of the disease; and the identification of the cause or associated medical conditions of ALI/ARDS [3].
### Calculation of the Lung Injury Score [LIS]

<table>
<thead>
<tr>
<th>Score</th>
<th>Chest Radiograph</th>
<th>Hypoxaemia Score</th>
<th>PEEP Score [when mechanically ventilated]</th>
<th>Lung Compliance [when available]</th>
<th>The score is calculated by adding the sum of each component and dividing by the number of components used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No alveolar consolidation</td>
<td>$P_aO_2/F_iO_2 \geq 300$</td>
<td>$\leq 5 \text{ cmH}_2\text{O}$</td>
<td>$\geq 80 \text{ ml/cmH}_2\text{O}$</td>
<td>No lung injury</td>
</tr>
<tr>
<td></td>
<td>Alveolar consolidation confined to 1 quadrant</td>
<td>$P_aO_2/F_iO_2 \ 225-299$</td>
<td>6-8 cmH$_2$O</td>
<td>60-79 ml/cmH$_2$O</td>
<td>Mild to moderate lung injury</td>
</tr>
<tr>
<td></td>
<td>Alveolar consolidation confined to 2 quadrants</td>
<td>$P_aO_2/F_iO_2 \ 175-224$</td>
<td>9-11 cmH$_2$O</td>
<td>40-59 ml/cmH$_2$O</td>
<td>Severe lung injury</td>
</tr>
<tr>
<td></td>
<td>Alveolar consolidation confined to 3 quadrants</td>
<td>$P_aO_2/F_iO_2 \ 100-174$</td>
<td>12-14 cmH$_2$O</td>
<td>20-39 ml/cmH$_2$O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alveolar consolidation extended to 4 quadrants</td>
<td>$P_aO_2/F_iO_2 &lt; 100$</td>
<td>$\geq 15 \text{ cmH}_2\text{O}$</td>
<td>$\leq 19 \text{ ml/cmH}_2\text{O}$</td>
<td></td>
</tr>
</tbody>
</table>

| 0 | 1 | 2 | 3 | 4 |

### Table 1-2 Calculation of Lung Injury Score [LIS] proposed by Murray et al [3].

#### 1.1.1.3 AECC ARDS Criteria

In 1992, the American European Consensus Conference [AECC] was convened to define the criteria for ARDS. They recommended the term “acute respiratory syndrome” instead of “adult respiratory syndrome” because ARDS is not limited to adults. The
AECC proposed the following diagnostic criteria for ARDS: (a) acute onset; (b) arterial hypoxaemia resistant to oxygen therapy alone; (c) bilateral lung infiltrate on chest radiograph; and (d) pulmonary artery wedge pressure ≤ 18mmHg when measured or absence of clinical evidence of left atrial hypertension. Importantly, the AECC proposed two disease severity levels based on oxygenation impairment: acute lung injury [ALI] to define a subset of patients with a \( \frac{P_aO_2}{FiO_2} \) ratio less than 300 mmHg; and ARDS to describe sicker patients with a ratio less than 200 mmHg [Table 1-3] [4].

The clinical and scientific impact of the AECC can be best demonstrated by the fact that from 1994 many clinical studies have been carried out based on these common criteria and led to changes in clinical practice, such as lung protective ventilation [5].

<table>
<thead>
<tr>
<th>Definition of Acute Respiratory Distress Syndrome and Acute Lung Injury</th>
</tr>
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<tbody>
<tr>
<td>• Acute onset</td>
</tr>
<tr>
<td>• Bilateral infiltrate on chest radiograph consistent with pulmonary edema</td>
</tr>
<tr>
<td>• Hypoxaemia</td>
</tr>
<tr>
<td>ALI: ( \frac{P_aO_2}{FiO_2} \leq 300 \text{ mmHg} )</td>
</tr>
<tr>
<td>ARDS: ( \frac{P_aO_2}{FiO_2} \leq 200 \text{ mmHg} )</td>
</tr>
<tr>
<td>• Absence of heart failure</td>
</tr>
<tr>
<td>No clinical evidence of left heart failure</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure ≤ 18 mmHg</td>
</tr>
</tbody>
</table>

Table 1-3 American European Consensus Conference diagnostic criteria for ARDS and ALI [4].

1.1.1.4 Berlin ARDS Criteria

Changing demographics of patients suffering from ARDS and the widespread availability of new investigations and technologies in medicine since 1994 has demanded the re-evaluation of the validity and reliability of the initial AECC ARDS definition. Main limitations of the AECC definition included the lack of exact description of the meaning of “acute”; the sensitivity of \( \frac{P_aO_2}{FiO_2} \) ratio in the context of varying PEEP and \( FiO_2 \); the poor reliability of chest radiography due to inter-observer variability and difficulties in distinguishing between hydrostatic and permeability pulmonary oedema. In 2012, an expert panel initiative of the European Society of Intensive Care Medicine [ESICM], the American Thoracic Society [ATS] and the Society of Critical Care Medicine [SCCM] convened to redefine ARDS based on a
robust meta-analysis of 4188 patients with ARDS [6]. Importantly, the populations were selected to represent wide range of facilities treating ARDS.

<table>
<thead>
<tr>
<th>The Berlin definition of ARDS [6]</th>
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<tbody>
<tr>
<td><strong>Timing</strong></td>
</tr>
<tr>
<td>• Onset of ARDS is within 1 week of a known clinical insult or new or worsening respiratory symptoms.</td>
</tr>
<tr>
<td><strong>Chest imaging</strong> [Chest radiograph or computed tomography scan]</td>
</tr>
<tr>
<td>• Bilateral opacities – not fully explained by effusion, lobar/lung collapse, or nodules</td>
</tr>
<tr>
<td><strong>Origin of oedema</strong></td>
</tr>
<tr>
<td>• Respiratory failure not fully explained by cardiac failure or fluid overload.</td>
</tr>
<tr>
<td>• Need objective assessment [e.g. ECHO] to exclude hydrostatic oedema if no risk factor present.</td>
</tr>
<tr>
<td><strong>Oxygenation</strong></td>
</tr>
<tr>
<td>• Mild: 200 mmHg &lt; PaO$_2$/FiO$_2$ ≤ 300 mmHg with PEEP or CPAP ≥ 5 cmH$_2$O</td>
</tr>
<tr>
<td>• Moderate: 100 mmHg &lt; PaO$_2$/FiO$_2$ ≤ 200 mmHg with PEEP ≥ 5 cmH$_2$O</td>
</tr>
<tr>
<td>• Severe: PaO$_2$/FiO$_2$ ≤ 100 mmHg with PEEP ≥ 5 cmH$_2$O</td>
</tr>
</tbody>
</table>

Table 1-4 The Berlin definition of ARDS[6].

The new consensus draft definition for ARDS introduced three severity categories based on the degree of hypoxaemia and removed the term ALI from the nomenclature. According to this, ARDS severity is defined as: (1) **mild ARDS** PaO$_2$/FiO$_2$: 200-300 mmHg; (2) **moderate ARDS** PaO$_2$/FiO$_2$: 100-200 mmHg; (3) **severe ARDS** PaO$_2$/FiO$_2$ ≤ 100 mmHg. The new definition also included the following criteria: (1) acute onset of ARDS, arising within a period of 7 days; (2) radiological evidence of ARDS. The use of chest computed tomography has been included and echocardiography is recommended to help to exclude pulmonary oedema secondary to cardiac causes. Consistent with current practice, which has seen a reduction in the use of pulmonary artery catheters, pulmonary artery wedge pressure as a variable was removed [Table 1-4]. Compared with the AECC definition, the new Berlin Definition had better predictive validity for mortality. According to this, **mild**, **moderate**, and **severe** ARDS were associated with increasing mortality; 27%, 32%, 45%, respectively; and increasing duration of mechanical ventilation in survivors.
1.1.2 EPIDEMIOLOGY AND OUTCOME FOLLOWING ARDS

ARDS confers an enormous disease burden, constituting the leading cause of death in paediatric and adult critical care, and exerts a substantial impact on public health care [7]. A recent prospective study from the United States reported a crude incidence of 78.9 cases per 100,000 persons per year, estimating 190,600 new cases of ALI per year, with an associated mortality rate of 40%, amounting to 75,000 deaths annually in US alone [The King County Lung Injury Project] [8]. In a European study carried out in Denmark, Sweden and Iceland, the reported incidence of ARDS and ALI was 31.4 per 100,000 person per year [9]. Based on the rapid growth of the population, Rubenfeld et al suggested that the incidence will likely to double in the next 25 years [8].

In spite of the many advances in the acute care of ALI/ARDS, significant morbidity is seen in 50-70% of survivors [7, 10] and the financial burden on society is considerable. The total health care related spending on the condition represent only a fraction of the total economic burden of the illness because many survivors are unable to resume their previous occupational activities. Hopkins et al reported that about one third of ARDS survivors in the US return to full time work or school, one third receive disability benefits and one third retire or do not work again [11].

Long term follow-up studies have shown that while pulmonary function does improve substantially at 3-6 months after ARDS, most ARDS survivors continue to exhibit altered diffusion capacity and mild restrictive pulmonary disease [10, 12, 13]. However, the persistent functional limitation resulting in reduced quality of life in survivors seems to be more strongly related to neuromuscular and cognitive impairment than to the pulmonary dysfunction per se [10, 14-17]. Psychiatric and psychological morbidity after ARDS is also considerable, including depression, anxiety and post traumatic distress syndrome [11, 18, 19]. Most importantly, recent studies have suggested that hypoxaemic and hypoglycaemic episodes at the early stage of critical illness may influence long term outcome domains, such as cognitive function and mood disorders [16, 17, 20, 21]. Currently, the mechanisms underlying long term consequences of ARDS are largely unknown. However, as the acute care of ARDS improves, the population of patients with long term sequelae will rise. Further
investigations into the mechanisms of emotional, physical and neurocognitive dysfunction are necessary to prevent these sequelae from undermining new developments in the acute care of ALI/ARDS.

1.1.3 AETIOLOGY OF ARDS

1.1.3.1 Pulmonary versus extrapulmonary ARDS

In general, the causes of ALI/ARDS can be separated into those that directly injure the lungs [pulmonary causes] or those that indirectly injure the lungs [extrapulmonary causes] [Table 1-5]. Direct ALI accounts for approximately 55% of all ALI cases, indirect ALI for 20% and further 21% appear to be due to mixed factors. The remaining 4% have no distinctive underlying pathophysiology [22]. Furthermore, pulmonary and extrapulmonary sepsis are the most common causes of ALI, with severe sepsis accounting for 46% of direct and 33% of indirect ALI cases [8, 23]. It is important to notice that, although the clinical symptoms of ARDS are shared by these aetiological factors, the underlying pathophysiological processes leading to lung injury are diverse. The implication of this is paramount, since understanding the diverse mechanisms that lead to ARDS may provide a firm ground to develop therapeutic strategies and cure patients [23, 24].

The histological and biochemical alterations seen in experimental direct and indirect ALI have been investigated by many [23, 25-27]. This can be illustrated best by Menezes et al’s work where intraperitoneal administration or direct instillation of E.Coli endotoxin to the lungs resulted in distinct histological and biochemical injury patterns in spite of similar changes in lung mechanics [26]. Direct lung injury caused more extensive damage to the alveolar epithelium [both to alveolar type I and II cells] and was associated with increased alveolar neutrophil accumulation and increased fibrinous exudates. In contrast, in indirect lung injury, histological injury was limited and primarily affected the lung endothelium and the interstitial space [permeability oedema formation]. Neutrophil apoptosis was also more dominant in direct lung injury compared to indirect injury. Cytokine levels [IL-6, IL-8, IL-10] were much higher in direct pulmonary insult than in indirect lung injury. Extracellular matrix remodelling [an important step in the resolution of ARDS] has been also investigated in the context of
the origin of the injury. It seems that collagen production is more intensive and occurs earlier in direct injury than in indirect injury [25, 26].

Significant research effort has been invested to determine the radiographic differences associated with direct and indirect injury. Chest radiograph and CT analysis of these two subgroups of patients led to the recognition that direct and indirect injury might be truly two separate entities. In general, this hypothesis was supported by the following morphological observations: (1) the radiological picture in pulmonary ARDS is dominated by patchy densities [representing pulmonary consolidation] and air bronchograms while (2) extrapulmonary ARDS express more “diffuse” or “hazy” densities [interstitial oedema and/or compression atelectasis]. However, the radiological picture in real life is more complex since the clinical presentation is often mixed [25].

Observations on lung mechanics in patients with direct and indirect lung injury further confirm the assumption that these might be separate entities. Gattinoni et al demonstrated first that the contribution of lung and chest wall elastance - determining the total respiratory elastance - depends on the origin of the lung injury. Patients with direct ARDS have higher lung elastance, while patients with indirect ARDS have increased elastance related to a stiffer chest wall [due to increased intra-abdominal pressure] [28]. These differences further explain why higher PEEP levels [>15 cmH\textsubscript{2}O] may result in more alveolar overdistension in patients with direct ARDS and, conversely, more alveolar recruitment in indirect ARDS [28]. Lower PEEP levels [<10 cmH\textsubscript{2}O] however appeared to be more beneficial in extrapulmonary ARDS. Similarly, indirect ARDS seems to be more responsive to prone positioning in terms of oxygenation than direct ARDS [25].

In summary, understanding the underlying mechanism of various ARDS phenotypes may be the key to devise specific therapies and management strategies. The heterogeneity in the aetiology of ARDS may explain why the majority of results from experimental research and clinical trials of therapeutic or supportive interventions failed to show overall survival benefits in ARDS [except the ARDSnet small versus high
tidal volume trial [ARMA] [[5]], or conversely, resulted only in some advantages in certain ARDS subgroups.

<table>
<thead>
<tr>
<th>Direct Lung Injury</th>
<th>Indirect Lung Injury</th>
</tr>
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<tbody>
<tr>
<td>• Pneumonia</td>
<td>• Sepsis</td>
</tr>
<tr>
<td>• Aspiration</td>
<td>• Multiple trauma</td>
</tr>
<tr>
<td>• Pulmonary contusion</td>
<td>• Acute pancreatitis</td>
</tr>
<tr>
<td>• Pulmonary embolism [fat, amniotic fluid, gas]</td>
<td>• Transfusion of blood products</td>
</tr>
<tr>
<td>• Near-drawing</td>
<td>• Cardiopulmonary bypass</td>
</tr>
<tr>
<td>• Inhalational injury</td>
<td>• Drug overdose</td>
</tr>
<tr>
<td>• Reperfusion induced lung injury</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-5 Summary of causes of pulmonary and extrapulmonary lung injury. Most common causes are pneumonia and systemic sepsis, followed by aspiration and trauma.

1.1.3.2 Sepsis versus non-septic causes of ARDS

Multiple studies suggest that non-septic causes of ARDS, particularly trauma, are associated with lower mortality than ARDS of infectious origin [8, 29-32]. Also, critically ill patients with non-septic causes have less chance to develop ALI than patients with sepsis [32, 363]. Indeed, severe sepsis, whether pulmonary or systemic in origin, is the leading cause of death in critically ill patients. The incidence of sepsis-induced critical illness is 150 per 100,000 person-years in the United States alone [31]. Evidence suggests that approximately 40% of patients with severe sepsis develop ARDS [32], and it has been estimated that sepsis-associated ARDS has an incidence of 45–63 cases per 100,000 person-years [33].

It is important to notice that demographic characteristics and physiological parameters also vary according to clinical risk factors for ALI/ARDS [8, 34]. Patients with aspiration or sepsis are generally older [with more co-morbidities] than patients with trauma [29, 34]. In addition, age-specific incidence of ALI and associated mortality increases with steeper frequency in sepsis than in non septic cases [8]. Patients with trauma related ALI have higher blood pressures, while septic patients [pulmonary or non-pulmonary origin] have lower blood pressures with higher prevalence of vasopressor use [29, 34]. This is associated with highest APACHE III scores in sepsis or non-trauma causes.
compared to trauma [29, 34]. Also, PaO$_2$/FiO$_2$ ratio seems to be the highest in trauma patient compared to other aetiologies [sepsis, pneumonia aspiration, others].

The apparent difference in epidemiological data in disease severity and outcome between septic and non-septic ALI patients led to the implementation of many clinical studies to investigate the inflammatory response in humans with ARDS. These studies looked at circulating or BAL biomarkers to demonstrate whether these markers correlate with specific clinical risk factors and their outcome in ARDS. The majority of studies focused on differences in septic and non-septic subjects or traumatic and non-traumatic inflammation.

In a small prospective cohort study, Moss et al compared endothelial cell activation in patients with trauma and sepsis induced ARDS. The authors found that circulating von Willebrand factor [vWF], intercellular adhesion molecule-1 [ICAM-1] and E-selectin levels were significantly higher in septic patients than trauma patients on admission as well as at the onset of ALI/ARDS. Importantly, ICAM-1 and E-selectin levels - surrogate markers of endothelial activation - were comparable to normal values in the trauma group. In spite of a few limitations to the study, this was the first time that a difference between ALI subgroups has been demonstrated on the inflammatory level. Later, Ware et al showed in a small retrospective study that protein C levels are significantly lower and thrombomodulin levels are higher in pulmonary oedema fluid from ALI patients with sepsis than those without sepsis. The authors also observed that low levels of protein C were associated with worst clinical outcome across both groups, including in hospital mortality and duration of unassisted ventilation. Subsequently, three larger studies evaluated the data from the ARMA [35, 36] and ALVEOLI trials [29] [Table 1-6]. Although, some of the data are conflicting among these studies [29, 36], overall these observations suggest that there might be a different inflammatory pattern depending on the precipitating factor of ARDS, i.e. sepsis being associated with higher inflammatory pattern than non-septic causes.
Study | Method | N | Results
--- | --- | --- | ---
Eisner et al\(^{[35]}\) | Secondary analysis of ARMA | 565 | SP-D is highest in sepsis and lowest in trauma induced ARDS.
Parsons et al\(^{[36]}\) | Secondary analysis of ARMA | 861 | Baseline IL-6, IL-8, IL-10 were significantly higher in sepsis or pneumonia than in patients with other clinical risk factors.
Calfee et al\(^{[29]}\) | Secondary analysis of ARMA and ALVEOLI | 1451 | Trauma patients with ALI had lower levels of: ICAM-1, SP-D, vWF, sTNFr-1 compared to non-trauma patient.

**Table 1-6** Results from the secondary analyses of the ARMA and ALVEOLI trials. N: number of patients involved in the analysis. SD-P: Surfactant protein D; sTNFr-1: soluble TNF receptor 1.

### 1.1.4 PATHOGENESIS AND PATHOLOGY OF ARDS

Although the cause of ALI/ARDS includes a broad range of diseases, the most fundamental characteristic of the illness is the *disruption of the alveolar-endothelial barrier*\(^{[37]}\). The clinical symptoms of the resulting protein rich permeability oedema is severe hypoxaemia and decreased pulmonary compliance. The first pathological description of lungs from patients with ALI described three phases with distinct ultrastructural changes\(^{[38]}\). The acute, *exudative phase* [first 1-6 days] is characterised by diffuse interstitial and alveolar oedema, with accumulation of neutrophils, macrophages and red blood cells, and the presence of eosinophilic hyaline membrane [fibrin, plasma proteins, cellular debris] in the alveoli. Both the epithelial and endothelial cells are injured and there is an extensive denudation of epithelial cells from the basement membrane. In the subacute, *proliferative phase* [7-14 days], some of the oedema fluid is reabsorbed [mainly from the alveolar space] and the gradual appearance of respiratory type II cells over the injured alveolar surface dominates the histological picture. Importantly, there is fibroblast infiltration and some evidence of collagen deposition at this stage of the injury. The course of ARDS in some patients may progress to a “chronic” or *fibrotic phase* [over 14 days]. Cells dominating the structural picture are mainly lymphocytes and alveolar macrophages. The balance between fibrosis and alveolar epithelial repair will eventually determine which patients recover completely or progress to a syndrome of fibrosing alveolitis and chronic respiratory failure.
Injury to the lung can occur by several mechanisms. However, one of the critical final steps in the pathogenesis of ALI is **neutrophil sequestration** that ultimately leads to the damage of the alveolar-epithelial barrier [39, 40]. Activation of lung endothelium, epithelium and residential alveolar macrophages leads to increased levels of cytokines in blood or alveolar space or both [TNF-α, IL-6, IL-8, IL-1β, MIP2-α, ICAM-1, VCAM-1, etc.], promoting neutrophil recruitment via adhesion and transendothelial migration to the alveolar space [40]. Neutrophil derived mediators, such as reactive oxygen species [ROS], proteases, cationic peptides and matrix metalloproteases [MMP] are essential in host defense, but equally can cause alveolar epithelial damage and organ dysfunction. The central role of neutrophils in tissue damage and immune defense can be best demonstrated by preclinical studies whereby neutrophil depletion reduced endotoxin and hemorrhage induced ALI [41], while in bacterial infection induced ALI it impaired bacterial clearance [42]. Importantly, in parallel to the acute inflammation, anti-inflammatory mediators are released [IL-10, IL-4, IL-13, IL-1RA, TGF-β] counteracting the cytokine storm caused by the initial insult. Reparative processes also become activated to facilitate early tissue repair [40, 43-45]. The link between the initial signals and the subsequent responses in lung cells [endothelial, epithelial, neutrophil and residential macrophages] is coordinated by transcription factors which trigger genes central to both inflammation and tissue repair. One of the key transcription pathways involved in this process is the nuclear factor kappa B [NF-κB] pathway, which has been described as the “master regulator of inflammatory responses” [46].

**1.2 MANAGEMENT OF ARDS**

**1.2.1 MECHANICAL VENTILATION IN ARDS**

The only intervention to convincingly demonstrate a significant reduction in mortality in patients with ARDS/ALI is “lung protective” ventilation [5], i.e. mechanical ventilation strategies that reduce lung stretch. This was a result of a series of conceptual shifts based on accumulated knowledge and insights into the role of mechanical ventilation in the development of ALI.
1.2.1.1 Traditional approaches to mechanical ventilation

Traditionally, most patients with respiratory insufficiency requiring mechanical ventilation received high tidal volume ventilation [10-15 ml/kg]. Peak inspiratory pressure of 50 cmH2O was considered acceptable quite often in the absence of barotrauma [47]. The main aim of this strategy was to maintain normal physiological indices in blood gas parameters. From the 1990s onward clinicians began increasingly to recognize that high intensity lung ventilation [traditional ventilation modes], restoring normal physiologic values, may be harmful and the invasive ventilation itself was potentially injurious in ARDS [48, 49]. In an early study, Rouby et al demonstrated that mechanical ventilation can be injurious by causing pneumothorax, pneumomediastinum or other manifestations of barotrauma [47]. More importantly, invasive ventilation causes microscopic damage with histological appearances similar to early ARDS [50].

1.2.1.2 Ventilation induced lung injury

Originally, Ashbaugh et al recognized the link between application of PEEP and lower mortality rate in patients with ARDS [1]. Later, Webb and Tierney demonstrated in an in vivo rodent model that high inflating pressures damages the lung and, in fact, PEEP, can prevent ventilation induced lung injury [51]. In the 1980’s the use of computed tomography in ARDS clearly demonstrated that lung injury was not uniformly affecting the lung tissue. Most importantly, it has become clear that in ARDS large part of the lung does not take part in gas exchange and that the actual volume of the healthy lung is very small [52]. This led to the concept of “baby lung” as a functional entity and to the recognition, that overdistension of this smaller lung during mechanical ventilation was causative of VILI [53, 54]. Furthermore, the aetiological role of atelectasis in the development of lung injury led to the routine application of PEEP in ARDS [1, 50, 51, 53-55]. PEEP increases the functional residual capacity [FRC] of the lung by recruiting collapsed alveoli. This in turn, increases the size of the “baby lung” and reduces the risk of overdistension. Application of PEEP also redistributes alveolar lung water, improving ventilation perfusion inequality [V/Q]. In addition, PEEP may prevent cyclic opening and closing of alveoli, thereby reducing atelectrauma [51]. However, application of
PEEP, particularly when the size of the “baby lung” is small may result in overdistension and haemodynamic instability [52].

The recognition that distorting forces generated during mechanical ventilation - overdistension and atelectasis - can induce a series of intracellular events, resulting in gene expression and transcription of pro-inflammatory mediators, led to the concept of biotrauma. Increased level of inflammatory mediators triggered by mechanical ventilation however can contribute to further damage in ALI/ARDS and by “leaking” to the systemic circulation may cause distal organ damage and may drive multi organ failure [MOF] [56, 57].

1.2.2 CURRENT MANAGEMENT STRATEGIES

1.2.2.1 Lack of specific therapies in ARDS

Despite ongoing intensive research, there are no specific therapies for ALI/ARDS. Up to date the cornerstone of ARDS management is mainly supportive and limited to protective mechanical ventilation [5] and restrictive intravenous fluid management [58]. Recently, a study using neuromuscular blockade in the early phase of severe ARDS have shown 90 days survival benefit in the treatment group with increased time of ventilator free days without increasing muscle weakness [59].

Although substantial amount of preclinical data contributed to elucidate some of the underlying molecular mechanism of ALI/ARDS, clinical studies using pharmacological agents failed to convincingly demonstrate improved outcome after ALI/ARDS. Pharmacological therapies that have been tested without success include pulmonary vasodilators, such as nitric oxide [NO] [60, 61], anti-oxidants [62, 63], and surfactant [64]. Similarly, corticosteroids [65] and immunomodulatory agents such as granulocyte-macrophage colony stimulating factor [66] failed to improve outcome in ALI/ARDS. These observations raise the question as to whether specific treatments may perform better in more homogenous cohorts of patients and highlights the need to understand the differences in pathophysiological processes in various ARDS aetiologies.
1.2.2.2 Supportive strategies for ARDS

Lung protective ventilation. Mechanical ventilation with low tidal volumes and low inflation pressure has been shown to reduce mortality in ARDS. This has been supported by three randomized trial [5, 67, 68]. The largest, the ARMA trial, demonstrated a 9% absolute reduction in mortality in patients ventilated with 6 ml/kg V_t compared to 12 ml/kg V_t ventilation [5]. In 2006, another multicentre study from Spain [ARIES study] had to be stopped due to significantly better survival in the low tidal volume and PEEP group [V_t: 5-8ml/kg and PEEP above the lower inflexion point + 2 cmH_2O] than in the higher tidal volume and PEEP group [V_t: 9-11 ml/kg and PEEP ≥ 5 cmH_2O]. Based on these observations and the substantial amount of preclinical data, low tidal volume ventilation with a V_t range of 4-8ml/kg and plateau pressure below 30 cmH_2O is recommended for patients with ARDS.

Permissive hypercapnia. Decreasing the intensity of mechanical ventilation in order to reduce the potential for stretch induced lung injury results in hypercapnia, which has been termed “permissive hypercapnia”. Clinicians have increasingly adopted these ventilation strategies and become adept at managing hypercapnia in critically ill ARDS patients. Furthermore, a substantial number of preclinical studies and a secondary analysis of the ARMA study have led to a paradigm shift from “permissive” to “therapeutic” hypercapnia, suggesting that elevated CO_2 might be an independent therapeutic measure in the management ALI [69-73].

PEEP. Although there is a general agreement among experts that some amount of PEEP is beneficial, the optimal level of PEEP that should be used in patients with ALI/ARDS or the most appropriate method to titrate PEEP have not been determined. Up to date, three large clinical trials, controlled for V_t [both control and treatment groups received 6 ml/kg tidal ventilation], failed to prove mortality benefit in the high PEEP ventilation group [74-76]. Importantly, the LOVS [Lung Open Ventilation Study] [75] and the ExPress [76] trials demonstrated important reductions in both the incidence and mortality rate of refractory hypoxaemia in the high PEEP group. In addition, in the ExPress trial the number of ventilator free days was also higher in the
high PEEP group. Taken together, these results suggest that high PEEP ventilation may be beneficial in a subset of patients with severe ARDS.

**Other ventilatory supports.** Clinical studies on the application of HFOV in adults with ARDS have been limited to case series or small studies. Two RCTs have been carried out over the last decade to compare conventional ventilation [CV] with HFOV in adults [77, 78]. Neither of the studies could demonstrate survival benefit. However, in the study of Bollen et al, the post hoc analysis revealed favourable outcome with HFOV in a subgroup of patients with the most severe hypoxaemia [78]. Most recently, two major, multicenter, randomized trials questioned the advantage of HFOV over protective lung ventilation [365, 366]. In the Canadian OSCILLATE study [n=571], early institution of HFOV in moderate-severe ARDS patients was associated with higher in-hospital mortality in spite of significantly less refractory hypoxemia in the HFOV group [365]. Furthermore, in the Oscillation in ARDS [OSCAR, n=795] trial [366], the authors found no difference in 30-day mortality between HFOV and protective lung ventilation strategies. The poor outcomes in the OSCILLATE trial may have been related to haemodynamic compromise associated with high mean airway pressures applied in the HFOV group which was reflected in increased fluid and vasopressor requirement. Importantly, in both trials, patients in the HFOV groups received more sedatives and more neuromuscular blocking agents which perhaps also contributed in the outcomes. These studies indicate that patients may require more individualized therapy in ARDS. HFOV may be more advantageous in patients with more homogenous injury and more recruitable lung units [indirect or extrapulmonary ARDS] than in heterogenous and non recruitable lung injury [direct or pulmonary ARDS]. Application of HFOV in ARDS based on the currently available information therefore demands cautious consideration and is not recommended routinely.

Although extracorporeal membrane oxygenation [ECMO] has not clearly been demonstrated to improve outcome compared to current standard ventilation management of adult ARDS, experiences arising from ECMO studies and observations from the H1N1 influenza outbreak, such as the Extracorporeal Life Support Organisation [ELSO] registry [364], Australia and New Zealand ECMO H1N1 experience [79] and the UK randomised trial [CESAR] [80] indicate that referral to a specialized...
centre with ECMO experience should be considered early in patients with most severe forms of ARDS.

1.3 VENTILATION INDUCED LUNG INJURY [VILI]

1.3.1 MECHANISM OF INJURY IN VILI

1.3.1.1 Barotrauma versus volutrauma

In 1974 Webb and Tierney carried out the first in vivo study that demonstrated that mechanical ventilation using high peak airway pressures leads to lung injury. In their experiments rats were exposed to mechanical ventilation with 14, 30 and 45 cmH₂O peak inspiratory pressure [51]. No abnormality was found after 1 hour ventilation with 14 cmH₂O peak airway pressure; however, both 30 and 45 cmH₂O peak airway pressures were associated with moderate and severe pulmonary edema. These observations subsequently have been replicated in larger animals, including rabbits, sheep, dogs, and pigs [50, 81, 82]. Although the term barotrauma was commonly used in relation to VILI, Dreyfuss et al later suggested that high tidal volume ventilation may be a more important determinant of lung injury than high airway pressure per se, and coined the term volutrauma [83]. These observations have been later reported in rabbit and lamb models of VILI [81, 82].

1.3.1.2 Stress-strain relationship

Gattinoni et al later proposed a new approach, saying that, given the elastic property of the lung and chest wall, the injury mechanism of VILI can be better understand with the “stress-strain” relationship which well characterizes the behavior of elastic materials under distorting forces [84]. In this framework, transpulmonary pressure is the generating force, i.e. stress, and the corresponding volume change is tissue deformation, i.e. strain. Since these two components cannot function independently from each other [cause and effect], the original concepts of baro- and volutrauma are essentially the descriptions of the same distending force.

1.3.1.3 Atelectrauma

There is considerable evidence indicating that ventilation at low tidal volumes may also contribute to lung injury. Lung atelectasis may develop through diverse mechanisms:
(1) compression of lung tissue [mainly in the dependent part of the lung]; (2) reabsorption of air from occluded distal lung units or from low V/Q areas; (3) impaired surfactant function. The consequences of such processes during ALI/ARDS, are decreased compliance [increased work of breathing or need for higher transpulmonary pressure to be able to achieve target Vt ventilation]; impaired oxygenation [increased shunt fraction]; increased pulmonary vascular resistance [right ventricular dysfunction and increased microvascular leak], and direct lung injury as a result of repetitive opening and closing of distal lung units, termed atelectrauma [50, 55, 85]. Conversely, reopening of collapsed lung areas, either with recruitment maneuvers or application of PEEP, have been shown to reduce lung injury secondary to atelectasis [51, 54].

1.3.1.4 Biotrauma

The proposed biotrauma hypothesis states that positive pressure mechanical ventilation via mechanical stress/stretch and shear forces, even in the absence of severe structural lung damage, can alter cellular function and lead to the release of pro-inflammatory mediators, altered tissue repair/remodeling and apoptosis. Furthermore, lung-born mediators leaking to the systemic circulation may cause distal organ damage and contribute to the development of multiple organ dysfunction in critically ill patients [56, 57, 86, 87].

The main experimental approaches to support the biotrauma hypothesis have arisen from: (1) preclinical VILI models, whereby direct stretching of lung cells and the lung itself results in increased cytokine response [56, 86, 88-92]. Conversely, studies demonstrating that protective ventilation, e.g. application of PEEP, decrease pulmonary and systemic inflammatory cytokine levels [93]. (2) In vivo, ex vivo, in vitro demonstration of stretch related transcription factor activation associated with inflammatory gene expression [94-98]. (3) In vivo studies demonstrating that lung parenchymal cells can alter their transcriptional program in response to mechanical stretch using in situ hybridization, gene expression profiling and global genome analysis to identify VILI-related genes [56, 86]. (4) data from clinical studies demonstrating that protective lung ventilation is associated with decreased levels of serum pro-inflammatory mediators compared to conventional ventilation [36, 99].
1.3.2 MOLECULAR MECHANISMS UNDERLYING VILI

1.3.2.1 Mechatransduction

Mechanosensors. The biological response to cyclic stretch during mechanical ventilation occurs through *mechanosensors* which transmit signals from the deformed extracellular matrix to the interior of the cell. These can be transmembrane receptors, such as *integrins*, *stretch-activated ion channels* [SA-channels] or the *cytoskeleton* itself [87, 100].

**Intracellular signal transmission.** The main intracellular signaling cascades involved in mechanosensing are the *MAP-kinase pathways* consisting of a range of enzyme complexes including: *ERK-1/ERK-2*, *JNK-1/JNK-2* and *p38* [87, 92, 94]. The importance of these pathways in the pathogenesis of VILI is that they control essential cell functions, such as inflammation, cell proliferation, and programmed cell death. The fate of lung cells during mechanical stretch, therefore, depends on the final activation patterns of these pathways, i.e. signal divergence, convergence and amplification. MAP kinases eventually activate various *transcription factors* via phosphorylation, which upon activation translocate to the nucleus, bind to a specific promoter of DNA sequences, and initiate gene transcription. The transcription factors that are central to gene expression during mechanical stress include: *NF-κB*, *AP-1*, *C/EBP* and *NF-IL6* [87, 101].

1.3.2.2 Role of NF-κB pathway in mechanical stretch induced cell activation

Activation of the NF-κB pathway has been demonstrated to mediate stretch induced lung injury [95-97]. *Tremblay et al* demonstrated first in an *ex vivo* model of VILI that mechanical ventilation can cause direct induction of gene expression that leads to cytokine release [56].

Important insights from more recent studies into the casual relationship between mechanical stretch and lung inflammation via NF-κB pathway can be summarized as follows: (1) *In vitro* and *in vivo* studies have shown that increasing mechanical stretch causes increased cytokine production [IL-8]. Also, more prolonged exposure to cyclic stretch is associated with higher cytokine production over time [92, 95] (2) Mechanical
stretch results in MAP kinase dependent [JNK, ERK and p38] NF-κB pathway activation. This effect is abrogated by using pharmacological inhibitors of the MAP kinase cascade [92, 98]. (3) Excessive mechanical stretch causes NF-κB activation and NF-κB dependent cytokine release. This has been demonstrated in an ex vivo perfused rabbit lung model, whereby low V_t [6 ml/kg] ventilation with excessive high PEEP or zero PEEP [ZEEP], resulting in over-distension or alveolar instability, led to increased p50/65 binding to DNA, while optimal PEEP was associated with less NF-κB activation [97]. Most recently, Liu et al demonstrated in an in vivo mouse model that high tidal volume ventilation [30 ml/kg] caused time dependent NF-κB activation [increased phosphorylation of p65] and translocation, and this was associated with increased BAL TNF-α and PAI-1 levels, increased microvascular permeability and myeloperoxidase [MPO] activity compared to non-ventilated and low V_t ventilation [6 ml/kg] [96]; (4) less mechanical stretch [i.e. low V_t ventilation], although to a lesser extent, activates the NF-κB pathway indicating that mechanical ventilation, even in the “protective” range, is pro-inflammatory [96]; (5) cytokine mRNA expression activity [IL-8] is present within 30 minutes in lung epithelial cells in response to mechanical stretch and this is preceded by IκB-α degradation and NF-κB translocation [95]. This data indicates that transcription factor activation and gene expression may occur very early after the initiation of mechanical ventilation.

Overall, mechanical stretch can induce multiple intracellular pathways that eventually converge and activate gene expression [Table 1-7]. This is important, since as opposed to sepsis or other aetiological factors in ALI, we know that VILI starts right at the initiation of mechanical ventilation. Therefore, the effort to minimize the possibility of a second hit injury arising from a supportive intervention is extremely important. Understanding the contribution of the above discussed intracellular mechanisms of VILI is crucial to avoid such damage to the lungs in ALI/ARDS.
<table>
<thead>
<tr>
<th>Study</th>
<th>model</th>
<th>Cell/species</th>
<th>Transcription factor</th>
<th>Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pugin(^{[91]})</td>
<td>in vitro</td>
<td>human alveolar macrophages</td>
<td>(NF_{-}\kappa B)</td>
<td>TNF-(\alpha), IL-6, IL-8, MMP-9</td>
</tr>
<tr>
<td>Li(^{[98]})</td>
<td>in vitro</td>
<td>human type II alveolar epithelial cells</td>
<td>(NF_{-}\kappa B), AP-1</td>
<td>IL-8</td>
</tr>
<tr>
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<td>ex vivo</td>
<td>rabbit lung</td>
<td>(NF_{-}\kappa B), AP-1, CREB</td>
<td>-</td>
</tr>
<tr>
<td>Nin(^{[95]})</td>
<td>in vitro</td>
<td>A549 epithelial cells</td>
<td>(NF_{-}\kappa B)</td>
<td>IL-8</td>
</tr>
<tr>
<td>Liu(^{[98]})</td>
<td>in vivo</td>
<td>mice lung</td>
<td>(NF_{-}\kappa B), AP-1</td>
<td>TNF-(\alpha), PAI-1</td>
</tr>
</tbody>
</table>

**Table 1-7** Summary of preclinical experiments on intracellular cell responses to mechanical stretch.

### 1.3.3 EFFECTS OF VILI

As a result of injurious ventilation [barotrauma, volutrauma, atelectrauma and biotrauma] specific pathophysiological changes occur in the lung. These include: (1) **pulmonary edema**; (2) **mechanical stress failure**; (3) **distant organ dysfunction**.

#### 1.3.3.1 Pulmonary edema

Pulmonary edema formation in VILI relates to: (1) alterations mainly in microvascular permeability [alveolar-epithelial barrier disruption due to mechanical stress failure and inflammatory injury]; (2) to lesser extent, increased microvascular transmural pressure [hydrostatic edema] both in the extra-alveolar, and alveolar vessels due to increased V\(_t\) ventilation [as a result of mechanical interdependence]; and (3) surfactant dysfunction [50, 102-104].

#### 1.3.3.2 Mechanical stress failure: interdependence

Mechanical ventilation in diseased lungs with a non-homogeneous distribution of lesions may be subjected to greater regional stress than uniformly inflated lungs [90]. *Mead et al* proposed that if the lung is unevenly expanded, forces arising from transalveolar pressures [alveolar pressure minus pleural pressure] may vary considerably in different lung areas [105]. Traction forces exerted on collapsed alveoli
by surrounding expanded lung areas facilitate re-expansion of these units [mechanical interdependence]. However, the stress generated between collapsed and expanded lung units can result in potentially harmful stretching forces at the interface, affecting both the collapsed and expanded alveoli. Post mortem histological analysis of lung tissue of patients with ARDS ventilated with high \( V_t \) \([\sim 12 \text{ ml/kg}]\) and high PIP \([\sim 56 \text{ cmH}_2\text{O}]\), showed multiple expanded cavities and intraparenchymal pseudocysts \([0.8-4\text{mm}]\) mainly in areas adjacent to inflammatory and atelectatic parenchyma, suggesting that these forces may play a significant role in ventilation induced lung injury [47].

Both endothelial and epithelial cells can be exposed to mechanical stress failure during VILI. West and colleagues showed, that capillary transmural pressures exceeding 50 cmH\( _2\)O \([\sim 40 \text{ mmHg}]\), cause disruption of the capillary endothelium and alveolar epithelium in the rabbit lung. The investigators calculated that in this situation capillary wall stress can be as high as it is seen in the wall of the aorta \([8.2 \times 10^5 \text{ vs. } 8.7 \times 10^5 \text{ dyn/cm}^2, \text{lung capillary vs. aortic wall stress}]\]. Importantly, this stress can be further aggravated during high \( V_t \) ventilation due to increased longitudinal forces acting on the blood vessels, resulting in more injury in ALI/ARDS [106-108]. Gajic et al showed that short term ventilation with high \( V_t \) ventilation causes lung epithelial stress failure evidenced by the presence of large proportion of propidium iodide positive cells on fluorescent images [44]. Interestingly, the authors also observed that the plasma membrane defects were reversible and repaired in 60% of the cells, and only a small fraction of wounded cells underwent necrosis.

### 1.3.3.3 Distant organ dysfunction

The hypothesis that mechanical ventilation itself may induce distant organ injury and have a significant contribution in MOD has attracted significant research efforts. Clinical trials demonstrating reduced serum cytokine and chemokines levels [36, 99, 109], less organ dysfunction [110] and improved mortality [5] associated with low \( V_t \) ventilation suggested that there might be a causative relationship between mechanical ventilation and distant organ injury. Also, it has been reported that renal failure is more common in patients ventilated with conventional strategies compared to lung
protective ventilation [5, 110]. The mechanism by which VILI exerts its effect on the end organs remains to be elucidated [111]. One of the possible mechanisms is increased epithelial cell apoptosis in distant organs [86]. A landmark study by Imai et al showed that injurious ventilator strategies in an in vivo rabbit model of acid induced ALI significantly increased epithelial cell apoptosis in kidney and small intestine, increased serum and BAL cytokine levels over time [IL-8, MCP-1: monocyte chemotactic protein 1, GRO: growth-regulated oncogen], and worsened kidney function [112]. Plasma obtained from animals ventilated with injurious ventilation induced apoptosis in LL-RKC1 cells [rabbit renal proximal tubular cells] in vitro and this effect was reversed by co-administration of soluble Fas-ligand inhibitor [Fas ligand Ig]. The group also found a significant correlation between plasma soluble Fas-ligand concentration, conventional ventilation strategy and renal dysfunction in ARDS patients [112].

1.3.4 STRATEGIES TO MINIMIZE VILI

1.3.4.1 Insights from preclinical studies

Historically, ventilation induced lung injury in clinical terms has been synonymous with barotrauma; a serious and often life threatening complication of mechanical ventilation [47]. However, the realisation that mechanical ventilation may worsen pre-existing lung injury and may be a separate entity and causative factor in ALI/ARDS was not clearly recognized until the early 1970s [51]. The concept and the description of the main characteristics of VILI were stemmed from substantial amount of preclinical studies [50, 101]. The collected findings of these studies can be summarized as follows:

1) Excessive volume or pressure control ventilation causes obvious macroscopic and microscopic changes in lungs, such as pulmonary oedema, haemorrhage, disruption of the alveolar arterial membrane, severe alveolar epithelial and endothelial damage, hyaline membrane formation, interstitial oedema and polymorphonuclear [PMN] cell infiltration. Injuries arising from alveolar overdistension have been repeatedly demonstrated in small and large animal species in both in vivo and ex vivo experiments [51, 82, 113-119]. Conversely, prevention of overdistension by reducing the intensity of ventilation attenuated or prevented the development of VILI.
Application of PEEP also reduced VILI in a series of experiments in various animal species by improving compliance, reducing alveolar oedema formation and by preserving surfactant function [50, 51]. However, excessive PEEP may increase end inspiratory lung volume [overall lung distension] and worsen VILI.

One of the important observations from these studies is that the evolution of VILI is exponential rather than linear. The global threshold airway pressure at which injury starts to accelerate can be generalized across the different species and it seems to be between 30-40 cmH$_2$O. The clinical implication of this is twofold: (1) clinical studies defined protective inflation pressures below these values resulted in improved mortality [5, 67]; (2) higher alveolar pressures may be present in patients with ARDS even during protective ventilation due to the uneven distribution of the disease and to the “baby lung” phenomenon [52, 54, 105].

The importance of the nearly 30 years of accumulated knowledge in experimental research is highlighted in the fact that numerous clinical trials have been initiated and resulted in changes in clinical practice in mechanical ventilation [5, 53, 54, 69].

1.3.4.2 Insights from clinical studies

Hickling et al demonstrated first in a retrospective, and later in a prospective study that limitation of peak inspiratory pressure in patients with severe ARDS might be beneficial [48, 49]. Despite evidence from experimental studies, the results from the first four clinical studies in the late 1990’s, using lower tidal volume ventilation with limited plateau or peak inspiratory pressure, were conflicting [67, 120-122]. Subsequently in 2000, the ARMA trial, including 861 patients, showed a 9% absolute reduction in mortality rate in patients ventilated with a tidal volume $V_t$ 6 ml/kg and plateau pressure [$P_{plat}$] of less than 30 cmH$_2$O compared with $V_t$ 12 ml/kg and plateau pressure [$P_{plat}$] of less than 50 cmH$_2$O [5]. The difference in outcome between the positive and negative trials, at least in part, can be explained by the differences in the methodology [Table 1-8]. This can be summarized: (1) differences in the control groups between positive and negative trials, (2) small sample sizes in the first 4 trials, (3) large variability in patients demographics, such as age, aetiology of ARDS and single vs. multiple organ failure.
<table>
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<th>Benefit</th>
<th>$p_aCO_2$ [mmHg]</th>
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<td>Protective</td>
<td>60</td>
<td>7.0±0.7</td>
<td>50</td>
<td></td>
<td>54.5 ± 15.0</td>
<td>22.2 ± 3.9</td>
</tr>
<tr>
<td>Brochard et al 1998</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58</td>
<td>10.3 ± 7.7</td>
<td>38</td>
<td>No</td>
<td>41.0 ± 7.5</td>
<td>31.7 ± 6.6</td>
</tr>
<tr>
<td>Protective</td>
<td>58</td>
<td>7.1 ± 1.3</td>
<td>47</td>
<td></td>
<td>59.5 ± 19</td>
<td>25.7 ± 5.0</td>
</tr>
<tr>
<td>Brower et al 1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26</td>
<td>10.2 ± 0.1</td>
<td>46</td>
<td>No</td>
<td>40.0 ± 1.6</td>
<td>30.6 ± 0.8</td>
</tr>
<tr>
<td>Protective</td>
<td>26</td>
<td>7.3 ± 0.1</td>
<td>50</td>
<td></td>
<td>50.3 ± 3.5</td>
<td>24.9 ± 0.8</td>
</tr>
<tr>
<td>ARDS Network 2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>429</td>
<td>11.8 ± 0.8</td>
<td>40</td>
<td>Yes</td>
<td>35.8 ± 8.0</td>
<td>34.0 ± 9*</td>
</tr>
<tr>
<td>Protective</td>
<td>432</td>
<td>6.2 ± 0.9</td>
<td>31</td>
<td></td>
<td>40.0 ± 10</td>
<td>26 ± 7*</td>
</tr>
</tbody>
</table>

Table 1-8 Overview of study design and findings of the five major RCTs involving comparison of low versus high tidal volume ventilation. Control: high tidal volume group; Protective: low tidal volume group; n: number of patients included; $V_t$: tidal volume; $p_aCO_2$: arterial carbon dioxide. *: in these trials differences between plateau pressures between control and low tidal volume ventilation was larger than in the three negative trials.

Application of PEEP has been incorporated and is part of current ventilatory management to minimize VILI. However, the magnitude of the optimal PEEP is still the subject of active debate [See section 1.2.2.2]. This may relate to the fact that the main determinant of VILI is end respiratory lung volume rather than the actual tidal volume or FRC. Employing high PEEP at higher lung volumes or in the situation of very small “baby lung” may cause overdistension and may place the lung above the upper inflection point of the pressure-volume [PV] curve [123]. Furthermore, it is unknown what is the optimal PEEP that keeps the lung open and on the steep part of the PV curve; i.e. between the lower and upper inflection points. Also, surrogate markers - apart from plateau pressure - to measure actual lung volumes at the bedside are limited in clinical practice. Lastly, it seems that the aetiology of ARDS is an important factor [25, 28] and clinical studies looking at subgroups of patients ventilated with targeted, group specific PEEP may deliver the answer to this question.
1.4 HYPERCAPNIC ACIDOSIS IN ARDS

1.4.1 RATIONALE FOR PERMISSIVE HYPERCAPNIA IN ARDS

Traditional approaches to manage hypercapnia in adults with ARDS have focused on the potential deleterious effects of elevated CO₂. Hypercapnia has been associated with adverse outcomes in diverse clinical contexts, such as cardiac arrest, severe sepsis and raised intracranial pressure [124]. However, this approach has been questioned in the early 1990s, when clinical studies suggested that high intensity ventilation in patients with ALI may be more harmful than the potential risks arising from higher CO₂ levels [48, 49]. Protective lung ventilation allowing higher arterial CO₂ levels – termed “permissive hypercapnia” - in patients indeed improved outcome in ARDS [5, 67, 68]. Consequently, permissive hypercapnia became an integral part of mechanical ventilation strategies in critical care patients. The recognition that permissive hypercapnia may provide additional protection in ARDS, independently from low stretch ventilation [69], resulted in a paradigm shift and led to the concept of “therapeutic hypercapnia” [71, 72]. This hypothesis was backed up with a substantial amount of preclinical studies suggesting that hypercapnia is protective in diverse experimental ALI/ARDS models [Table 1-9][42, 88, 89, 125-132]. However, most recent studies, from our group and others, questioned the safety of HCA in the context of bacterial infection and repair indicating that the effect of hypercapnia is more complex and needs more understanding before its clinical application as a therapeutic measure [Table 1-9] [133-137].
<table>
<thead>
<tr>
<th>Study</th>
<th>Animal model</th>
<th>Applied CO₂ level</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shibata et al 1998[129]</td>
<td>free radical ex vivo (rabbit)</td>
<td>25 %</td>
<td>Protective</td>
</tr>
<tr>
<td>Laffey et al 2000[128]</td>
<td>pulmonary IR ex vivo (rabbit)</td>
<td>12 %</td>
<td>Protective</td>
</tr>
<tr>
<td>Broccard et al 2001[88]</td>
<td>VILI ex vivo (rabbit)</td>
<td>targeted paCO₂ [70-100 mmHg]</td>
<td>Protective</td>
</tr>
<tr>
<td>Sinclair et al 2002[89]</td>
<td>VILI in vivo (rabbit)</td>
<td>~12 %</td>
<td>Protective</td>
</tr>
<tr>
<td>Laffey et al 2003[127]</td>
<td>mesenteric IR in vivo (rabbit)</td>
<td>dose response curve [0, 2.5, 5, 10, 20%], final experiment 5%</td>
<td>Protective</td>
</tr>
<tr>
<td>Laffey et al 2003[125]</td>
<td>VILI in vivo (rabbit)</td>
<td>12%</td>
<td>Protective</td>
</tr>
<tr>
<td>Laffey et al 2004[126]</td>
<td>endotoxin in vivo (rat)</td>
<td>5%</td>
<td>Protective</td>
</tr>
<tr>
<td>NicChonghaile et al 2008[42]</td>
<td>E.Coli pneumonia in vivo (rat)</td>
<td>5%</td>
<td>Protective</td>
</tr>
<tr>
<td>Wang et al 2008[132]</td>
<td>fecal peritonitis in vivo (sheep)</td>
<td>targeted paCO₂ [55-65 mmHg] ~4.1%</td>
<td>Protective</td>
</tr>
<tr>
<td>Costello et al 2009[131]</td>
<td>CLP sepsis in vivo (sheep)</td>
<td>5%</td>
<td>Protective</td>
</tr>
<tr>
<td>Pellitekova et al 2010[138]</td>
<td>VILI in vivo (mouse)</td>
<td>dose response curve [0, 5, 12, 25 %] final experiment 12 %</td>
<td>Protective</td>
</tr>
<tr>
<td>Nichol AD 2010[139]</td>
<td>endotoxin in vivo (rat)</td>
<td>5%</td>
<td>Protective</td>
</tr>
<tr>
<td>Wu et al 2012[140]</td>
<td>pulmonary IR ex vivo (rat)</td>
<td>5%</td>
<td>Protective</td>
</tr>
<tr>
<td>O’Croinin 2005[141]</td>
<td>E.Coli pneumonia in vivo (rat)</td>
<td>5%</td>
<td>No effect</td>
</tr>
<tr>
<td>O’Croinin 2008[133]</td>
<td>prolonged E. Coli pneumonia [48h] in vivo (rat)</td>
<td>8%</td>
<td>Harmful</td>
</tr>
</tbody>
</table>

**Table 1-9** Summary of **in vivo** and **ex vivo** experiments on the effects of hypercapnia in diverse acute lung injury models. IR: ischaemia-reperfusion.
1.4.2 PHYSIOLOGIC EFFECTS OF HYPERCAPNIA

1.4.2.1 CO₂ transport

CO₂ is carried in the blood in three forms: dissolved, as bicarbonate [HCO₃⁻], or in combination with proteins as carbamino compounds [mainly as carboxy haemoglobin: COOH-Hb]. In general, CO₂ freely diffuses across cell membranes and in the presence of H₂O and carbonic anhydrase [CA] it becomes carbonic acid and subsequently dissociates to HCO₃⁻ and H⁺. HCO₃⁻ diffuses out and Cl⁻ moves into the red cells to maintain electrical neutrality [chloride shift]. Since in the deoxygenated blood Hb is in a reduced form, some of the H⁺ ions are transported as HHb [Haldane effect]. In the venous blood CO₂ transport further facilitated by the reaction between CO₂ and reduced Hb forming COOH-Hb [142].

1.4.2.2 Effects on the respiratory system

A large number of experimental studies attest that low-moderate hypercapnia [FiCO₂: 2-5%] improves arterial oxygenation both in normal [143-145] and diseased lungs [146, 147]. This is related to reduced alveolar-arterial oxygen difference [DAaO₂], V/Q heterogeneity, dead space and decreased regional perfusion heterogeneity. In contrast with this, two human studies reported increased shunt fraction and impaired oxygenation in patients with ARDS when permissive hypercapnia was achieved with low tidal volume [Vₜ] ventilation [148, 149]. A potential explanation for these disparate findings might lie in the fact that in the animal studies CO₂ was added to the inspired gas mixture, while in human studies hypercapnia was achieved by reduction of Vₜ. The increased shunt fraction and reduced oxygenation in the human studies may have been related to a reduction of Vₜ and airway closure rather than to hypercapnia per se. The effect of hypercapnic acidosis on airway resistance has been shown to be variable in humans [150, 151], while parenchymal lung compliance increases in response to hypercapnic acidosis due to increased surfactant secretion or more effective surface tension-lowering properties under acidic conditions [152].

1.4.2.3 Cardiovascular effects

Hypercapnic acidosis increases cardiac output despite an apparent direct inhibitory effect on myocardial contractility [153] and on vascular smooth muscle cells [70].
However, elevated CO\(_2\) activates the sympatoadrenal system and this seems to counteract the direct inhibitory effects of CO\(_2\). The net result is an increase in cardiac output [CO] [70, 154]. Hypercapnia also improves oxygen supply by increasing global oxygen delivery [increased CO and reduced intrapulmonary shunt fraction] and improving O\(_2\) offloading in the microcirculation [Bohr-effect]. At the same time, hypercapnia and acidosis seem to reduce O\(_2\) demand the periphery by shutting down cell metabolism [155].

1.4.2.4 Central nervous system effects

Hypercapnia is a potent ventilatory stimulant via peripheral and central chemoreceptors. Hypercapnic acidosis improves cerebral tissue oxygenation by augmenting PaO\(_2\) as well as cerebral blood flow [CBF] [156]. Hypercapnic acidosis causes precapillary cerebral arteriole dilatation, a function attributed to the acidosis rather than the hypercapnia per se [157]. Hypercapnic acidosis mediated increases in CBF are a clear concern in the setting of reduced intracranial compliance where increased global CBF may critically elevate intracranial pressure.

1.4.2.5 Effects on the immune system

Hypercapnic acidosis has a profound inhibitory effect both on the innate and the adaptive immune system. The mechanisms by which HCA exerts its effects may be related to hypercapnia itself or to the intra/extracellular pH changes during respiratory acidosis.

Innate immune system. The key cells initiating the inflammatory response to invading pathogens and endogenous signals are phagocytes: macrophages, neutrophils and dendritic cells. Their role is: (1) activation and recruitment of immune cells via cytokines; (2) amplification of the initial signal via cytokines and the complement system; (3) activation of the adaptive immune system via antigen presentation.

Cytokine production and HCA. Hypercapnia and acidosis interfere with the coordination of the immune response by reducing cytokine signaling between immune effector cells [158-161]. Acidosis reduces the production of key cytokines, such as TNF-\(\alpha\), IL-8 and IL-6 in neutrophils [158] and macrophages [161]. Neutrophil IL-8
production, which plays a vital role in modulating the acute inflammatory response, is reduced by hypercapnic acidosis [158]. The effect of hypercapnic acidosis on cytokine production can be sustained. This is evidenced by the fact that peritoneal macrophages were found to have reduced TNF-α production for three days following intra-peritoneal insufflation of CO₂ compared to controls [162].

**Microbial killing capacity, ROS production and HCA.** Catalytic enzymes responsible for production of reactive oxygen species [ROS] in neutrophils and macrophages have an optimum pH in which they function most efficiently. Therefore, ambient CO₂ levels may impact on their activity. Hypercapnic acidosis inhibits oxidant generation *in vitro* [158, 163, 164]. In the lung, hypercapnic acidosis reduces free radical induced tissue injury following ischemia-reperfusion [127, 128] and attenuates the production of NO metabolites [NOx] following both ventilation induced [88] and endotoxin induced [126] lung injury. Hypercapnic acidosis also inhibits xanthine oxidase mediated injury and directly inhibits the enzyme [129].

**Neutrophil function and hypercapnic acidosis.** Alterations in intracellular pH is also central to the regulation of normal neutrophil function, such as migration, chemotaxis, spreading, and normal apoptotic mechanisms [158, 164-166]. Persistent acid loading *in vivo* by permissive hypercapnia could overwhelm the capacity of neutrophils, and particularly in activated neutrophils it can result in abnormal function. The potential for HCA to attenuate neutrophil recruitment to the site of injury has been demonstrated in diverse experimental ALI models, such as ventilation [89] and endotoxin-induced [126] ALI.

**Monocyte and macrophage function.** HCA decreases the activity of both stimulated and unstimulated macrophages [136, 159-162]. HCA reduces endotoxin stimulated macrophage release of TNF-α and IL-1β *in vitro* [159, 161]. CO₂ pneumoperitoneum has also been demonstrated to impair peritoneal macrophage function [160].

**Adaptive immune system.** T and B cell mediated immune response is activated by the native immune system. Their function is: (1) to recognise foreign antigens in the presence of "self" antigens during the process of antigen presentation; (2) elimination of pathogens or pathogen infected cells; (3) development of immunological memory,
in which each pathogen is remembered by a specific antibody. These memory cells become activated and eliminate microbials more efficiently when subsequent infections occur.

Very little is known about the effect of HCA on these processes in the context of ALI. Most of the data arises from studies investigating the effect of HCA on tumour growth and spread. It seems that the optimal extracellular pH for tumour growth is significantly lower than in the normal tissue [pH: 6.8]. The main factor responsible for this is altered glucose metabolism in cancer cells resulting in increased H+ production. However, it has been shown that natural killer cell and lymphokine activated killer cells are inhibited at such pH environment and this may contribute to tumour spread [167].

1.4.3 INSIGHTS FROM CLINICAL STUDIES OF PERMISSIVE HYPERCAPNIA

1.4.3.1 ARDS

Hickling et al first proposed an alternative ventilation strategy to rescue patients with severe ARDS and to limit ventilation induced lung injury [48]. This incorporated: (1) lower peak inspiratory pressures [PIP] and low V_t ventilation; (2) application of PEEP; (3) acceptance of higher CO_2 levels. Hickling et al argued: “It seemed to us that an alternative approach would be simply limit PIP by reducing V_t and allow the CO_2 to rise. The PaCO_2 would be then stabilise at a new higher level and CO_2 elimination would be maintained at a lower level of alveolar ventilation, as occurs in patients with hypercapnia due to chronic obstructive airway disease.” Although the study had a number of limitations, the large difference in the predicted hospital mortality in favour of lung protective ventilation and permissive hypercapnia [16% vs. 39.6%] prepared the ground for future prospective studies [48].

Of the five prospective RCTs of protective ventilatory strategies published subsequently, two demonstrated an impact of ventilator strategy on mortality [5, 67], although three did not [120-122]. While to some extent, permissive hypercapnia developed in all of the trials, there was much variability among these studies [Table 1-8]. In addition, patients were not randomized to receive normocapnia or hypercapnia, since the primary aim of these studies was to investigate the effect of low stretch ventilation in ALI/ARDS. Therefore, although it is clear that ventilation strategy
can definitely impact on mortality - in the positive trials – there is no discernible relationship between levels of hypercapnia and survival among these data.

The database of the ARMA [5] has been subsequently analyzed to determine whether, in addition to the effect of tidal volume, there might also be an independent effect of hypercapnic acidosis [69]. Mortality was examined as a function of permissive hypercapnia on the day of enrolment using multivariate analysis and controlling for other co-morbidities and severity of lung injury. It was found that permissive hypercapnia reduced mortality in patients randomized to the higher tidal volume, but not in those receiving lower tidal volumes [69]. These are the first clinical data suggesting potential direct beneficial effects of hypercapnia in ARDS patients. However, although these clinical observations support a body of basic science on the beneficial effects of hypercapnic acidosis, they do not confirm them. Further appropriately designed randomized clinical studies are needed to elucidate the direct effect of permissive hypercapnia on ALI/ARDS.

1.4.3.2 Neonatal Respiratory Distress Syndrome [RDS]

Early observational studies from the 1980’s and 1990’s suggested that ventilation strategies involving permissive hypercapnia in premature newborns with ARDS lowers the risk for chronic lung disease [CLD]. Subsequently, Mariani et al reported in a small prospective study [49 patients] that in the first 96h of life ventilation strategies allowing higher PaCO₂ levels [45-55 mmHg] in preterm infants resulted in significantly faster weaning from mechanical ventilation [168]. Later, a larger multi-centre randomized trial [220 patients] of permissive hypercapnia and dexamethasone therapy including extremely low-birth weight infants were stopped because of unanticipated non-respiratory adverse events related to the dexamethasone therapy [169]. However, there was a trend towards a lower incidence of death and CLD in the permissive hypercapnia group [target PaCO₂ > 52 mmHg]. Furthermore, the need for assisted ventilation at 36 week gestational age was 1% in the permissive hypercapnia group and 16% in the control group. Finally, a prospective multicentre observational study from Denmark [407 patients] showed that ventilator strategy incorporating early use of nasal continuous positive airway pressure with permissive hypercapnia and
surfactant therapy significantly reduced the incidence of chronic lung disease in preterm infants [170]. Despite the many limitations of the studies, the significance of these data lies in the facts that the effect of permissive hypercapnia in two studies was directly investigated [168, 169], moderate hypercapnia [45-55mmHg] seemed to be safe and was associated with some benefits, although it did not improve mortality.

1.4.3.3 Asthma and Chronic Obstructive Pulmonary Disease [COPD]

Acute severe asthma is characterized by increased airway resistance, limited expiratory flow rate, premature airway closure and dynamic hyperinflation. Mechanical ventilation in this setting is a major concern due to increased risks for barotrauma and cardiovascular instability. Integration of controlled hypoventilation and permissive hypercapnia to the management of severe asthma was suggested first by Darioli and Perret in 1984 [171]. Subsequently two small observational studies demonstrated that decreasing minute ventilation [26, 16, 10 l/min], prolongation of expiratory time by decreasing respiratory rate [RR] and increasing inspiratory flow rate significantly reduced dynamic hyperinflation [172, 173]. Investigators in these studies intentionally did not correct hypercapnia and PaCO₂ was kept at moderately elevated levels [~ 63, 68 mmHg respectively]. Furthermore, it has been reported in a case series of severe status asthmaticus that hypercapnia over 150-200 mmHg and acidosis for several hours [~ 10h] have been tolerated well with no immediate or late adverse consequences [174]. Since acute severe asthma requiring mechanical ventilation is infrequent in clinical practice, the available evidence on the role of permissive hypercapnia in this subset of patients is limited. The acceptance of moderate hypercapnia however is routinely employed for patients with acute severe asthma admitted to the intensive care unit in Europe [175]. The rationale for the use of permissive hypercapnia in COPD is similar to that for acute severe asthma, i.e. it is permitted in order to minimize the potential for dynamic hyperinflation during mechanical ventilation. However, there are no clinical trials of permissive hypercapnia in COPD patients.
1.4.4 PRECLINICAL STUDIES OF HYPERCAPNIC ACIDOSIS IN NON-SEPTIC LUNG INJURY

1.4.4.1 Ischaemia-reperfusion lung injury

The clinical significance of lung ischaemia-reperfusion [IR] injury is attributed to (1) its central role in clinical contexts such as lung transplantation, pulmonary embolism, ARDS and multiple organ dysfunction [i.e. mesenteric ischaemia-reperfusion, reperfusion injury after aortic aneurysm repair or cardiac arrest etc.]; (2) its manifestation as a lung injury which may differ in direct and indirect IR injury characterized with distinct radiographic, morphologic and physiologic features [25, 28]; (3) inflammation during IR, such as cytokine release, activation of neutrophils and oxidative injury are prominent features of systemic inflammatory response syndrome that underlies MOF; (4) the systemic effects may provide insights into the mechanism of "cross-talk" between organs in multiple organ dysfunction syndrome.

The rationale to investigate the effect of HCA on ALI in the context of IR originated from early observations whereby metabolic acidosis was protective against IR lung [176] and myocardial injury [177]. In addition, hypercapnia also had been shown to attenuate hypoxic-ischaemic brain injury in vivo [178]. Shibata et al first reported, in 1998, that hypercapnic acidosis [HCA] is protective in an ex vivo free radical induced ALI model [129]. The authors used 25% CO₂ to test their hypothesis, however, such degree of hypercapnia and associated acidosis [pH: 6.84] would represent extreme clinical conditions and not the generally accepted “permissive hypercapnia” levels. Nevertheless, hypercapnic acidosis reduced capillary permeability by more than 50% in the injured lungs, possibly via inhibition of endogenous xanthine oxidase. Laffey et al further explored this area using both ex vivo and in vivo IR models. The authors concluded: (1) HCA is more protective than metabolic acidosis in pulmonary IR injury ex vivo [179]; (2) buffering HCA attenuates this protective effect [179]; (3) the effect of HCA is dose dependent [127]; (4) HCA ameliorates direct [128] and indirect [127] ALI induced by pulmonary and mesenteric IR in vivo. This was supported by improved oxygenation, lung permeability and lung mechanics under hypercapnic conditions. HCA also blunted cytokine release [TNF-α], lipid peroxidation, peroxynitrite production and reduced apoptosis in lung tissue. Most recently, Wu et al reported that moderate
hypercapnia [10%] attenuates pulmonary reperfusion injury in isolated rat lungs via NF-κB pathway inhibition [140].

1.4.4.2 Ventilation induced lung injury

The first study demonstrating that HCA directly reduces VILI was carried out by Broccard et al in an ex vivo rabbit lung model [88]. Isolated rabbit lungs were ventilated with either low PIP [15 cmH₂O] or gradually increasing PIP [20-25-30 cmH₂O] and exposed to normocapnia or HCA. The investigators found that HCA reduced microvascular permeability, lung oedema formation, and BAL protein content, however, the clearly depended on the severity of the mechanical injury. A subsequent study by Sinclair et al confirmed and extended these observations in an in vivo rabbit VILI model [89]. The question, how HCA affects VILI in a clinically more relevant ventilator strategy, has been subsequently explored by Laffey et al in an in vivo rabbit VILI model [125]. Ventilation with \( V_t \) 12ml/kg resulted in improved oxygenation and lung mechanics under hypercapnic conditions. Recently, Halbertsma et al reported that low \( V_t \) ventilator strategy [\( V_t \): 8 ml/kg] with PEEP [4cmH₂O] for 2h was associated with increased BAL neutrophil count and pro-inflammatory cytokines in an in vivo mouse model compared to non-ventilated controls. Administration of 2% or 4% CO₂ to the breathing circuit significantly attenuated mechanical stretch induced lung inflammation [180]. Most recently, Pletekova et al revisited the effect of HCA on inflammatory response in low and high \( V_t \) [12 vs. 45 ml/kg] ventilation in an in vivo mouse model [138]. HCA significantly improved oxygenation, lung elastance, microvascular permeability and lung histology in animals ventilated with the high \( V_t \) group, but not in the low \( V_t \).

Overall, the assumption that HCA is beneficial in the context of severe ventilation induced lung injury is clear. However, the subtle, intracellular effects of permissive hypercapnia or the effect on repair mechanisms during lung protective ventilation [i.e. at \( V_t \sim 6 \text{ ml/kg} \)] still needs to be elucidated. To demonstrate such effects, investigators may need different in vivo models or surrogate markers of injury and/or repair.
1.4.5 PRECLINICAL STUDIES OF HYPERCAPNIC ACIDOSIS IN SEPTIC LUNG INJURY

Protective ventilator strategies and consequent permissive hypercapnia are integral parts of the management of critical ill patients with severe sepsis. Although large number of experimental studies attests that hypercapnia is protective in various non infectious ALI models, most recent experimental studies suggest that hypercapnia may be deleterious in the context of sepsis induced ALI [133, 137, 181]. These concerns related to: (1) the potential of hypercapnia to inhibit the immune system; (2) the potential of hypercapnia to facilitate microbial growth; (3) the potential of hypercapnia to delay tissue repair and contribute in biotrauma. The fact that more and more patients survive the hyperacute phase of sepsis due to advanced intensive care management inevitably generated a patient population with persistent immune dysfunction/immunoparalysis, whom are extremely vulnerable to secondary or opportunistic infections. Therefore, therapeutic strategies or clinical states which promote or result in immunosuppression may be devastating in this patient population.

1.4.5.1 Hypercapnia/acidosis and bacterial growth

Environmental carbon dioxide can both stimulate and inhibit bacterial growth. Lower concentrations, as low as 0.25%, up to 20-60% can promote growth in various aerobic and anaerobic microorganisms [growth response depends on the particular bacterial strain], however higher concentration can inhibit or reduce growth rates [182, 183]. These effects have been exploited in microbiological culture techniques, where 5-10% CO₂ is commonly used to detect microorganisms from samples obtained from patients with sepsis or other infectious diseases. Conversely, in the food industry, high concentration of CO₂ [up to 100%] or application of pressurized CO₂ [2.5-25 MPa] is used to kill bacteria and prevent food spoilage [184, 185].

Pugin et al recently demonstrated that supernatants from cultured lung epithelial cells exposed to cyclic stretch - conditioned media - are acidotic and enhance bacterial growth compared to supernatants from un-stretched cells [186]. This effect was related to higher H⁺ concentration in the stretched group, since alkalisation of the culture media or inhibition of the Na⁺/K⁺ATPase enzyme reduced bacterial growth,
while acidification augmented it. The group also showed that the observed metabolic acidosis in the supernatant is proportional to mechanical stretch. A range of gram-positive and gram negative bacteria \([E. \, coli, \, Proteus \, mirabilis, \, Serratia \, rubidaea, \, Klebsiella \, pneumoniae, \, Enterococcus \, faecalis, \, and \, Pseudomonas \, aeruginosa]\) isolated from patients with ventilator-associated pneumonia showed increased proliferation in acidified media and the pH range optimal for bacterial growth was found to be between 6.0-7.2. Interestingly, pH of exhaled breath condensate from ventilated patients was significantly lower than in healthy subjects [6 vs. 7.14].

Although the above data describes the impact of two distinct situations on bacterial growth, i.e. the effect of \(CO_2\) and metabolic acidosis, these may have significant clinical implications. Institution of mechanical ventilation with permissive hypercapnia may favour for bacterial proliferation and present additional risks in recovery or in the development of secondary infections in intensive care patients.

1.4.5.2 Protective effect of HCA on the early phase of bacterial pneumonia - experimental studies

The effect of HCA in pneumonia induced ALI model appears to depend on the stage and the severity of the actual infection. Ni Chonghaile et al demonstrated in an acute \(E. \, coli\) pneumonia model that HCA was associated with less hemodynamic alterations, better oxygenation and smaller decrement in lung compliance compared to animals exposed to normocapnia [42]. Six hour exposure to HCA did not alter bacterial load in BAL, blood and lung tissue. The authors also hypothesized that HCA may exert its anti-inflammatory effect via inhibition of neutrophil function. Using a neutrophil depleted animal model, the group demonstrated that HCA caused less tissue injury and less bacterial growth than normocapnia [42]. These results suggest that the protective effect of HCA on tissue injury, at least in the context of evolving acute pneumonia, is independent from the presence of neutrophils. To see the effect of HCA in a more clinically relevant model, the group introduced HCA 6 hours after the induction of pneumonia [130]. HCA reduced the severity of lung injury compared to normocapnia as evidenced by better lung mechanics in the HCA group. Although the protective effect of HCA was modest, HCA did not increase bacterial load in BAL and blood. In contrast with this, O’Cronin et al, employing a less severe pneumonia model, could not
demonstrate the protective effect of HCA [141], indicating that the magnitude of the injury is an important factor in detecting the biological effect of HCA.

These findings suggest that acute HCA does not promote bacterial proliferation and is safe in early and more established phases of pneumonia. HCA also blunts tissue injury independently from inhibition of neutrophil cells.

1.4.5.3 Potential harmful effect of HCA in prolonged bacterial pneumonia

In the context of prolonged, untreated pneumonia, HCA may be deleterious. O’Croinin et al demonstrated in an E.coli pneumonia model that rats exposed to 48 hours of environmental hypercapnia had higher bacterial colony counts in the lung tissue and had more lung damage [133]. These were evidenced by worst static compliance and histological injury scores compared to normocapnic control animals. In addition, neutrophils isolated from hypercapnic animals had impaired phagocytic activity. Of importance to the clinical context, when antibiotic treatment was instituted the harmful effect of hypercapnia disappeared. Although, environmental hypercapnia induces hyperventilation in spontaneously breathing animals and could potentially worsen lung injury, the authors demonstrated no direct deleterious effects of environmental hypercapnia induced hyperventilation on lung function.

Overall, the importance of these data lies in the fact that this was the first time that hypercapnia proved to be harmful in the setting of sepsis induced ALI by increasing bacterial load in the lung.

1.4.5.4 Protective effect of HCA in systemic sepsis

Our group previously demonstrated that 3 and 6 hour exposure to HCA reduced the severity of early septic shock and sepsis induced lung injury and improved survival in a caecal ligation and puncture [CLP] model [131, 187]. This was evidenced by delayed development of circulatory shock, better central venous oxygen saturation, and ameliorated increase in serum lactate levels under hypercapnic conditions. HCA also improved oxygenation and lung mechanics, reduced lung permeability and decreased BAL neutrophil counts. BAL TNF-α and IL-6 levels were significantly reduced only in the
6 hour CLP group. Importantly, HCA did not alter BAL and peritoneal bacterial load. Of note, HCA reduced blood bacterial counts in the 6 hours series.

Wang et al used a more established faecal peritonitis induced sepsis model to investigate the effect of HCA on sepsis [132]. The authors randomized female sheep to receive HCA, dobutamine infusion or control conditions and followed them up until death [mean survival time ~18-19 hours]. HCA, similarly to dobutamine, increased heart rate, cardiac index and oxygen delivery, and reduced serum lactate levels in septic animals compared to normocapnia. Most importantly, HCA had a more favourable effect on lung injury than dobutamine, evidenced by reduced lung oedema formation, alveolar-arterial oxygen difference, shunt fraction, and BAL IL-6 levels.

These data strongly suggest that HCA is protective in septic shock and may reduce sepsis induced ALI in the early phase of sepsis. Also, HCA does not increase bacterial load, reassuring that hypercapnia is not harmful, at least in the early phase of sepsis.

1.4.6 OTHER CONCERNS REGARDING THE EFFECTS OF HCA

1.4.6.1 Repair following injury

Mechanical ventilation in ARDS produces alveolar trauma. This occurs due to over-distension of alveoli and alveolar wall shear forces due to atelectrauma. Electron microscopic images show widespread endothelial and epithelial injury characterized by plasma membrane blebs and cytoskeletal disruptions associated with inter- and intracellular gaps. These alveolar wounds have been demonstrated in experimental VILI [44] as well as in the clinical settings [108, 188]. In addition, it is well known that at least 50% of ARDS survivors experiences long term morbidity [7, 10], including pulmonary dysfunction. Reparative processes after ARDS, therefore, have long term implications in this patient population.

The possibility that HCA may impair wound healing in VILI was brought into focus by Doerr et al [189]. This group examined the effects of HCA on cell membrane disruption in ex vivo and in vitro models of VILI. They concluded that HCA impaired plasma membrane resealing under HCA conditions as compared to normocapnia [189]. Our group recently has investigated the effect of hypercapnia and acidosis in a series of in
vitro experiments [134]. These observations confirmed that: (1) HCA reduced the rate of wound closure in three different pulmonary cell types [SAEC: small airway epithelial cell, HBE: human bronchial cells, A549 lung epithelial cell]; (2) HCA inhibited wound closure in a dose dependent manner, severe acidosis [15% CO₂, pH 6.9] being the most inhibitory followed by moderate HCA [10% CO₂, pH 7.15]; (3) when buffering was instituted, hypercapnia delayed wound repair in A549 cells similarly to those had been seen with HCA; (4) normocapnic alkalosis did not alter wound repair compared with normocapnia; finally (5) delayed wound repair was related to inhibition of cell migration.

The biological effect of hypercapnia may be mediated by transcription factors, such as NF-κB. Takeshita et al have shown that HCA inhibits NF-κB activation via a reduction in the degradation of its inhibitory molecule - IκB-α - in the setting of endotoxin induced injury in pulmonary endothelial cells [190], thus providing a basis for the mechanism by which HCA attenuates ALI. Based on this observation, our group has demonstrated that HCA inhibited κB dependent transcription and IκB-α degradation induced by scratch wound compared to normocapnic conditions. Furthermore, IκB-α super-repressor transgene delivery [an inhibitor of NF-κB activation] delayed wound healing in a similar fashion as HCA.

These findings raise real concern about the biological effects of HCA and suggest that “permissive hypercapnia” might be deleterious in the reparative phase of ALI/ARDS. Considering the central role of NF-κB pathway in the facilitation of wound repair [134, 191, 192], it seems that inhibition of this pathway by hypercapnia may be harmful in the context of late ARDS and may have significant role in adverse outcomes in ALI/ARDS.

1.4.6.2 Alveolar fluid reabsorption

Removal of alveolar oedema fluid in ARDS is one of the key steps in the resolution of lung injury. This process is closely linked with active Na⁺ transport across the apical surface of alveolar epithelial type I and II cells [43]. As Na⁺ moves via epithelial Na⁺ channels into the cells, the Na⁺/K⁺-ATPase pump extrudes the absorbed Na⁺ on the basolateral side of the cell membrane creating an osmotic gradient across the cells.
This is followed by water absorption from the alveolar space [37, 40]. Since lung epithelial cells take active part in alveolar fluid clearance, direct injury to these cells – mechanical, inflammatory or oxidative damage – can significantly impair the ion transport system and delay the resolution of ALI [193]. This concept is supported by the fact that more than 50% of patients with ALI/ARDS develop impaired alveolar fluid clearance [194]. Furthermore, intact or repaired alveolar fluid reabsorption is associated with better survival during ALI/ARDS [194].

There is very little information regarding how hypercapnia or HCA influences alveolar oedema reabsorption. Briva et al reported in an ex vivo isolated-perfused rat lung model, that hypercapnia [independently from intra- and extracellular pH] reversibly and in a dose dependent manner reduced alveolar fluid removal by inhibiting Na⁺/K⁺-ATPase function [195]. This occurred through activation of the PKC [protein kinase C] ζ isotype, leading to phosphorylation of the α1 subunit of the Na⁺/K⁺-ATPase. The resulting endocytosis of the Na⁺/K⁺-ATPase pump was associated with reduced oedema clearance. Vadasz et al further investigated the putative intracellular mechanism of hypercapnia in a series of in vitro experiments. The authors found that elevated CO₂ rapidly [in a dose dependent manner] activated JNK [c-jun N terminal kinase] and led to decreased Na⁺/K⁺-ATPase pump activity. This effect was reversed by pharmacological inhibitors of JNK and adenoviral mediated over-expression of a dominant-negative variant of JNK. Furthermore, more proximal steps in JNK activation [phosphorylation] were also affected by elevated CO₂ and were associated with impaired Na⁺/K⁺-ATPase activity [196].

Although, the present data on the biological effects of hypercapnia and alveolar fluid clearance are very valuable insights, they raise few important questions: (1) it is important to know that in the ex vivo isolated lung model the lungs were not ventilated and CO₂ was added to the perfusate instead. Therefore, the primary target of CO₂ was the lung endothelium rather than the lung epithelium. It is unknown, however, whether this data can be extrapolated to the situations where CO₂ is added to the breathing circuit or it is simply not eliminated, such is the case during lung protective ventilation; (2) the authors used uninjured lung models, therefore it is unknown how hypercapnia or acidosis would affect alveolar fluid clearance in the
context of acute injury or repair; (3) although it is clear that intracellular pathways can be best investigated in in vitro models, the biological effects of hypercapnia in intact animal models may result in entirely different response. Nonetheless, these and other data [133, 134] convey the message that permissive hypercapnia may be harmful in the reparative phase of ALI/ARDS and more research is needed to elucidate the biological mechanism of CO$_2$ in this setting.

1.4.7 MECHANISMS OF ACTION OF HYPERCAPNIA

1.4.7.1 Acidosis versus hypercapnia

One question posed by investigators and clinicians alike is whether any effects seen in response to HCA are a result of hypercapnia per se or the acidosis associated with hypercapnia. Summarising and comparing the scientific data on this matter is difficult, mainly because of differences in methodology and the heterogeneity of applied experimental models. In general, one of the ways to test the separate effect of pH and CO$_2$ is to buffer respiratory acidosis. This is quite reasonable since buffering is a normal physiological reaction to acidosis and the on other hand administration of buffers remains a common practise in critical care. For example, three of the protective ventilation trials buffering with sodium bicarbonate were allowed although not used in all patients [5, 67, 122]. The evidence that the effects of hypercapnic acidosis in ARDS are a function of the acidosis rather than hypercapnia has been suggested by many. Laffey et al used sodium hydroxide to buffer respiratory acidosis and demonstrated that buffering worsened ischaemia-reperfusion injury in an ex vivo model [179]. Nichols et al allowed renal buffering in an in vivo model and demonstrated that buffered hypercapnia worsened lung injury in both endotoxin sepsis and E. coli pneumonia [181]. Caples et al have shown that buffering attenuated plasma membrane injury by using tris-hydroxymethylaminoethane [THAM] and sodium bicarbonate [NaHCO$_3$] in ex vivo and in vitro VILI repair models [197]. In contrast with this O’Toole et al, using NaHCO$_3$ as a buffer agent, suggested that the potential harmful effect of HCA on in vitro wound repair might be related to CO$_2$ rather than to the pH [134]. Others suggested that the effect of CO$_2$ on microbial killing and host defence is independent from intra- and extracellular pH changes in a series of in vitro experimental studies [135-137].
Application of buffered hypercapnia to investigate the contribution of acidosis and CO₂ to injury development inherently carries multiple problems. Firstly: physiological buffering of respiratory acidosis involves many buffer systems, including blood, kidney, interstitial fluid compartment and intracellular pH regulation, and their absolute or relative contribution to injury would be difficult to determine. Secondly: administration of bicarbonate infusion can paradoxically increase arterial CO₂ without substantial changes in extracellular pH and can alter intracellular pH. Thirdly: THAM buffering may be an option to avoid the unwanted effects of bicarbonate administration. However its role is not completely clarified in the management of acidosis in critically ill patients. Fourthly: there are gaps of knowledge in how various extracellular buffering would affect intracellular pH regulation and what the consequence of this on cell function.

Buffering respiratory acidosis in clinical practice remains a controversial issue. The main concern is that buffering may simply ablate any protective effects of acidosis [either metabolic or respiratory] while not addressing the primary problems. Administration of buffers that does not increase CO₂ load may be an option when buffering is considered [such as THAM] or alternatively, administration of CO₂ to the breathing circuit in the late phase of inspiration may create a situation when selective acidification of lung tissue coexist without the systemic effects of respiratory acidosis [145].

1.4.7.2 Hypercapnia and the NF-κB pathway

A large body of evidence attests that HCA suppresses inflammatory/immune response to injury [See 1.4.2.5]. The mechanisms by which hypercapnia exerts its effect are not entirely understood. However, recent data indicates that CO₂ may inhibit the NF-κB pathway. The first publication reporting the potential of HCA to attenuate endotoxin induced injury via an NF-κB dependent mechanism was published by Takeshita et al [190]. The authors demonstrated that HCA prevented IκB-α degradation in LPS stimulated pulmonary endothelial cells and this coexisted with decreased cytokine expression [ICAM-1, IL-8], reduced cell injury [reduced LDH in cell lysate supernatant] and reduced neutrophil adherence to the endothelial monolayer. More recently our group have demonstrated that the potential for HCA to reduce pulmonary epithelial
wound repair is also mediated via an NF-κB dependent mechanism [134]. Others proposed the existence of an intracellular CO₂ molecular sensor mediating these effects. Cummins et al have shown in 6 different cell lines that moderate hypercapnia [5-10% CO₂] facilitated the nuclear transport of IKK-α in a reversible manner, reduced the degradation of IκB-α in the cytoplasm and inhibited the translocation of p65 to the nucleus [135]. These events were independent from changes in extracellular or intracellular pH. Expression of NF-κB dependent pro-inflammatory genes [CCL2, ICAM1, TNF-α] were blunted, while anti-inflammatory genes, such as IL-10, were enhanced by 10% CO₂. Others have shown that elevated CO₂ suppressed host defence by inhibiting NF-κB dependent antimicrobial peptide gene expression in Drosophila resulting in increased mortality to bacterial infection [137]. High CO₂ levels, up to 20% also have been shown to inhibit IL-6, TNF-α induction and phagocytosis in LPS stimulated macrophages [136]. In the two later studies hypercapnia inhibited the NF-κB pathway without affecting IκB-α degradation, suggesting that other pathways or regulatory steps may have been involved in mediating the immunosuppressive effect of hypercapnia. These data suggest that both the beneficial and the deleterious effects of HCA are linked, at least partially, to the NF-κB pathway [See 1.5].

1.5 THE NF-κB PATHWAY IN ARDS

1.5.1 COMPONENTS OF THE NF-κB PATHWAY

NF-κB is a dimeric protein complex composed of different combinations of members of the “Rel” and “NF-κB” subfamily of transcription factors and is involved in DNA binding and transcriptional regulation [198, 199]. This protein family consists of five main molecules, called p50, p52, p65 [RelA], c-Rel and Rel B, and share an N-terminal homology domain [RHD] responsible for a nuclear localization signal, DNA binding, homo- and heterodimerization and interaction with inhibitory IκB proteins. NF-κB dimer is normally sequestrated in the cytoplasm of non-stimulated cells, bound to IκB [IκB-α, IκB-β or IκB-ε] inhibitory proteins. Signaling to NF-κB upon diverse stimuli proceeds through numerous intracellular pathways and activates the IκB-kinase complex [either IKK-α or IKK-β or IKK-γ]. It is important to know that upstream events via several interlinking signaling pathways that mediate IKK activation generate a
unique pathway that tailors the final NF-κB response specific to the actual inciting stimulus. The activated IKK complex subsequently phosphorylates IkB proteins on two N-terminal serine residues [Ser\textsuperscript{32} and Ser\textsuperscript{36}], which dissociates from NF-κB and is ubiquinated and degraded by the 26S proteosome. NF-κB then translocates to the nucleus and binds to specific cognate binding sequences [κB sites] in the promoter/enhancer regions of different inflammatory target genes where it initiates the transcriptional apparatus [Figure 1-1][200].

The basic scheme of NF-κB signaling consists of a series of regulatory elements starting with membrane receptors and receptor proximal signaling adaptor molecules, the IKK complexes, IkB proteins, NF-κB dimers and post-translational modifications [200, 201]. However, the key step for controlling NF-κB activity and signal responsiveness is the interaction between IkBs and NF-κB [202]. While the mechanisms that underlie the termination of NF-κB activity is not completely elucidated, it is generally accepted that the activated NF-κB increases the resynthesis of IkB-α protein resulting in a negative feedback. The resynthetized IkB-α, at least in part, terminates the transcription by retaining the NF-κB dimers in the cytoplasm. Other mechanisms, such as degradation of active NF-κB or displacement of NF-κB dimers from the DNA regulated by nuclear cofactors are newly described intracellular processes and require more understanding [201].
Figure 1-1 NF-κB signal transduction pathways. In the canonical [or classical] NF-κB pathway, NF-κB dimers such as p50/p65 are maintained in the cytoplasm in association with an independent IκB molecule [most often IκB-α]. The binding of a ligand to surface receptor [Toll like receptors- TLR4; IL-1R or TNFR1] recruit adaptor molecules, such as TRAF, Myd88, TRADD, IRAK4 to the cytoplasmic domain of the receptor. In turn, these adaptors recruit the IKK catalytic complex [IKK-α/β and NEMO, called IKK-γ] directly onto the cytoplasmic adaptor molecules. This clustering of molecules at the receptor site activates the IKK complex. IKK then phosphorylates IκB at two serine residues, which leads to its ubiquitination and degradation by the proteosome. NF-κB then enters the nucleus to turn on target genes. The canonical pathway is most commonly activated in response to cell injury due to diverse stimuli, such as mechanical stretch, LPS, bacterial exotoxin, cytokines or reactive oxygen species. In contrast, the non-canonical [alternative] pathway activates the p100/RelB complexes during B and T cell organ development. This pathway further differs from the canonical pathway because it activates an IKK complex which consists of two IKK-α subunits. IKK activation is mediated by NIK [NF-κB-inducing kinase] and eventually leads the ubiquitination and partial degradation of p100/RelB complex and the release of the p52/RelB molecules. [This figure was created and kindly donated by Claire Masterson]
1.5.2 ROLE OF THE NF-κB PATHWAY IN THE PATHOGENESIS OF ARDS

1.5.2.1 Evidence from clinical studies

Persistent elevation of pro-inflammatory cytokines is associated with adverse outcomes in ALI/ARDS. Donnelly et al found that BAL concentrations of IL-8 were significantly higher in patients who subsequently progressed to ARDS. The main source of IL-8 production in the lung appeared to be the alveolar macrophages [203]. Furthermore, persistently increased concentrations of BAL cytokines [IL-1β, TNF-α, IL-6, IL-8] have been correlated with ongoing ARDS and poor outcome in patients with sepsis [362].

Regulation of cytokine expression is under the control of diverse transcription factors, such as NF-κB [198, 199]. The promoter/enhancer region of each individual gene encoding a particular cytokine contains a transcription factor specific sequence [e.g., κB sequence for NF-κB] where the transcription factor is bound and controls gene expression [200]. Activation of transcription pathways - particularly the NF-κB pathway - therefore is central to the evolution of inflammatory response in ALI [See 1.5.2.2-1.5.2.3].

To establish the relationship between NF-κB activity and the severity/outcome of ARDS in humans is challenging. The following methodological approaches have been used in clinical studies to pin down this problem: (1) measurements of NF-κB pathway activity in either BAL or peripheral immune cells [macrophages or neutrophils]; (2) measurements of NF-κB pathway activity in response to stimuli [LPS or patient BAL fluid] in immune cells from BAL or blood in vitro; (3) stimulation of alveolar epithelial cell lines [e.g. A549] with BAL fluid from patients with ALI/ARDS and subsequent measurement of NF-κB activity in vitro. These clinical studies have shown that alveolar macrophages from patients with ARDS have higher NF-κB translocation and DNA binding activity than control ICU patients [204, 205]. Others have found that patients with ARDS who’s neutrophils responded to stimulation with less NF-κB translocation had better survival and more ventilator free days than patients with more responsive neutrophils [206]. Bronchoalveolar lavage from ARDS patients that contained higher amounts of neutrophils, IL-1β, IL-8, myeloperoxidase [MPO] and nitrated proteins
correlated with increased NF-κB binding activity in A549 cells in vitro [207]. Furthermore, BAL fluid stimulated alveolar epithelial cells and monocytes had higher NF-κB activity, and this correlated with ALI severity and BAL NOx and peroxynitrite content [208].

Although, these studies do not give direct information as to what is the exact contribution of NF-κB pathway in ARDS evolution and outcome, they provide important clues and directions in the understanding of the pathomechanism of lung injury.

1.5.2.2 Effects on inflammation

The contribution of lung epithelial cells and NFκB pathway in lung inflammation has been demonstrated by many groups [209-212]. Selective transduction of airway epithelial cells by adenoviral vectors containing either IKK-α or IKK-β transgenes resulted in constitutive activation of NF-κB, induction of mRNA expression of several cytokines [MIPs, INF-γ-inducible protein 10, and monocyte chemoattractant 1], increased level of BAL cytokines [MIP-2, KC] and increased neutrophil count [209]. Histological changes showed intra alveolar accumulation of neutrophils and alveolar septal thickening. Observations from this model indicated that activation of the NF-κB pathway in pulmonary epithelial cells can drive pulmonary inflammation and has a central role in the development of ALI. Others demonstrated that transgenic mice expressing dominant negative inhibitor of NF-κB translocation, the IκB-α super-repressor [IκBα-SR] in distal airway epithelium developed significantly less lung injury after inhaled LPS than in wild type animals [211, 212]. This was associated with reduced levels of myeloperoxidase, TNF-α, IL-1β, MIP-2, KC, and VCAM-1 in lung tissue and lesser histological injury. Similarly, NF-κB inhibition has been demonstrated to attenuate pulmonary ischaemia-reperfusion [213], and sepsis induced ALI in vivo [210]. However, in these models the lung endothelium rather than the epithelium was the primary site of injury.
1.5.2.3 Effects on injury resolution and repair

NF-κB up-regulation and resultant gene expression is equally important in cell protection, wound repair, and host defence. Selective ablation of IKK-β in intestinal epithelial cells in mice has shown to increase susceptibility to radiation and increased epithelial cell apoptosis in vivo [192]. Egan et al demonstrated in an in vitro intestinal epithelial [rat intestinal epithelial 1 (RlA-1)] scrape-wound injury model that cell migration and restitution, at least in part, depends on NF-κB activity [191]. Cells close to the wound edge had increased NF-κB activity, which co-existed with increased epidermal growth factor receptor activation [EGFR], a molecule that is important in the initiation of signalling cascades responsible for cell migration. Pharmacological inhibition of EGRF and NF-κB decreased cell migration by up to 50% and selective overexpression of IkB-α-SR inhibited by around 60%. Similarly, O’Toole et al reported that lung epithelial wound repair was inhibited by pharmacological inhibitors of the NF-κB pathway [PDTC and BAY 11-7085]. In addition, overexpression of IkB-α-SR transgene also inhibited wound healing [134] and this was related to impaired cell migration rather than reduced cell proliferation.

NF-κB activation may be crucial in normal host defence in the context of bacterial infection. It has been demonstrated in an in vivo P. aeruginosa pneumonia model that stimulation of NF-κB activity via constitutive overexpression of the RelA gene enhanced bacterial clearance [214], while inhibition of the pathway resulted in reduced neutrophil count and increased bacterial colony counts in the lung [215]. In a separate experiment the investigators also showed that the reduced bacterial clearance was associated with reduced oxidase-dependent respiratory burst activity in macrophages [214, 215].

1.5.3 MODULATION OF THE NF-κB PATHWAY – A DOUBLE EDGED SWORD?

The NF-κB pathway is one of the major mechanisms that coordinate both the pro-inflammatory and reparative processes in lung injury [216]. Inferences from preclinical data suggest that complete inhibition of the pathway may not be the answer to organ protection [45, 134, 135, 137, 191, 214, 215]. Inhibition of the NF-κB pathway may reduce ALI and protects against tissue injury, but at the same time it may attenuate
host response and delay organ repair. Theoretically, the consequence of complete NF-κB inhibition can be detrimental in critically ill patients by transforming the illness form acute to chronic. The key to success, however, lie in the understanding how NF-κB pathway regulates inflammation, and perhaps, more specific inhibition or temporary dampening of pathway activity could be the right approach for ALI [46].

1.6 GENE THERAPY FOR ARDS

1.6.1 RATIONALE FOR GENE THERAPY IN ARDS

There are several points which suggest that gene therapy has the potential to become an effective tool in the treatment of ARDS: (1) ARDS/ALI is an acute, but relatively transient process. Therefore, gene delivery and subsequent transgene expression may only need to persist for a relatively short period of time obviating the need for multiple dosing regiments with associated adverse immunological response. (2) The distal lung epithelium is selectively accessible through the airways via nebulisation technologies. (3) The pulmonary vasculature is also relatively accessible, as the entire cardiac output must transit this circulation. Promising strategies may involve the intravenous administration of modified delivery vectors that recognise antigens selectively expressed on the pulmonary endothelial surface. (4) Gene therapy may be an excellent method to explore the mechanism of ARDS - both the injury and reparative phases – in vivo by modifying key intracellular pathways. Finally (5) gene therapy based approaches offer the potential to selectively target differing phases of ARDS, e.g. by using promoters that allow genes to switch on or off during distinct phases of the disease process.

1.6.2 BARRIERS TO GENE THERAPY

Mechanical barriers. Results from preclinical and clinical studies suggest that the main hurdles to effective gene therapy are extracellular barriers [217, 218] and immunological responses to gene transfer [219]. The lung has evolved effective barriers to prevent the uptake of any inhaled foreign particles. These include the mucociliary system which traps and clears inhaled materials from the lung; the glycocalyx over the surface of epithelial cells; tight junctions; and limited particle uptake from the luminal site of the airway [217, 218]. In ALI/ARDS, the presence of
pulmonary oedema, fibrosis, atelectasis, cell debris and additional extracellular barriers such as mucus or sputum create even more obstacles for efficient gene delivery. Although, all these physical and molecular barriers may impede gene transfer, encouragingly, there is sufficient preclinical evidence for successful lung epithelial/endothelial transduction in ALI/ARDS.

**Immune system.** Barriers to gene transfer also include the innate immune system. Alveolar macrophages have been shown to be able to eliminate viral vectors via phagocytosis and also antigen presentation [219]. Furthermore, both single and multiple administrations of viral vectors can trigger non-specific - cytokine mediated-and specific inflammatory - CD8⁺/CD4⁺ - responses and can hinder efficient gene transfer [219]. Pre-existing immunity from antibodies to wild type viruses, such is the case with adenovirus [Ad] and adeno-associated virus [AAV], can eliminate recombinant viral vectors and interfere with efficient gene transfer [218, 220, 221]. Restricting vector administration to a single dose in ALI/ARDS may resolve these problems, however it also limits the therapeutic potential of gene delivery. Since immunogenicity strongly relates to viral capsid proteins, application of uncommon serotypes or engineering modified vector capsids can overcome these obstacles. Also, choosing targeted, spatially localised vector administration instead of a systemic approach may provide another option to avoid inflammatory response during gene therapy.

1.6.3 GENE DELIVERY SYSTEMS

Genetic materials are transferred by delivery systems to the target tissue. The ultimate aim is to construct a vector system that is safe, efficient and can be easily administrated to the patient. Transfer systems can be classified to viral and non viral vectors systems. This section briefly discusses the most commonly used vectors to date in preclinical and clinical studies.

1.6.3.1 Viral vectors

**Adenoviral vectors.** Adenoviruses contain a double stranded DNA genome. The major advantage of this vector system is their relative ease of production [218] and the high efficiency at which they can transduce pulmonary epithelial cells. The main
disadvantage of adenoviral vectors, however, their ability to trigger the immune system, particularly after repeated doses. This is related to the fact that the majority of the population have been exposed to adenoviruses in their childhood. In order to reduce host immune response, newer, third-generation adenoviral vectors have been generated, called “gutless” or “helper-dependent” vectors, containing only DNA sequences that are responsible for packaging and DNA synthesis [217]. Adenoviral vectors have been used to successfully transduce injured lungs in preclinical studies [See 1.6.4] [222, 223] and are well-tolerated at low to intermediate doses in humans [224].

**Adeno-associated viral vectors.** Adeno-associated virus [AAV] vectors have a good safety profile, broad tissue tropism, long duration of expression, and may have superior capacity to escape from immune system surveillance compared with other viruses [220, 225]. The packaging capacity of the virus is limited to ~4.7 kilo base pairs [kbp]. Several clinical trials have been carried out in the nose and lungs of cystic fibrosis [CF] patients, all using the AAV2-based vector CFTR [cystic fibrosis transmembrane conductance regulator] transgene construct, which was shown to have an excellent safety profile [226-229]. Different AAV subtypes produce different capsids that bind to specific receptors on the apical membrane of the lung epithelium [See Chapter 8, section 8.2.3.3]. Among the different serotypes, AAV-5 and AAV-6 exhibit the best tissue tropism for the pulmonary epithelium. To date, transduction by AAV to the lung has been carried out in humans [See Chapter 8, section 8.2.2], Rhesus macaques [230], rabbits [231], dogs [232] and rodents [232, 233, 234, 344].

**Lentiviral vectors.** Lentiviruses are a subclass of Retroviruses and belong to the RNA virus family [235]. Unlike other retroviruses, lentiviruses are able to transfec non dividing somatic cells. Virus replication requires reverse transcriptase enzyme to translate the genetic information from the RNA to DNA. The main feature of the virus its stability and reliable expression capacity [~100 days], however, it has a tendency to randomly integrate into the host genome. Random integration may carry a risk of activation of oncogenes and may cause malignant transformation in somatic cells [236]. Prolonged gene expression with lentiviruses, might be advantageous in chronic
diseases, such as cystic fibrosis, while in ARDS this feature might be deleterious [218, 235].

1.6.3.2 Non viral vectors

Non-viral approaches to somatic gene therapy also exist, including the use of naked DNA/RNA oligonucleotides, plasmid DNA and DNA-complexes. These approaches are generally less efficient than viral vectors in transducing lung epithelium, but the advantage of these approaches are that they are less likely to induce an immune response [218]. This is an important factor for inflammatory disease, such as ARDS, particularly if repeated administration is required.

Plasmids. Plasmid vectors are double-stranded circular DNA segments. Gene delivery to the cell with plasmid vectors or naked DNA is highly inefficient unless the DNA is associated with other molecules or physical energy is applied to temporarily disrupt the targeted cell membrane [218]. Since plasmid DNA contain no proteins for attachment to cellular receptors, cellular uptake can occur only if the plasmid is in close contact with the desired cell type [See Chapter 8, section 8.3.8]. Electroporation, ultrasound, pressurised vascular delivery, laser, magnetic fields and “gene guns” have been used to facilitate plasmid delivery [237]. As plasmid DNA appears to be safe, it seems that plasmids with physically enhanced delivery will be used increasingly in clinical trials.

DNA complexes. DNA complexes are DNA segments surrounded by a lipid coat [lipoplexes] or other polymers, such as polyethyleneimine [polyplex] which facilitates cellular adhesion, uptake and endocytosis [237]. Synthetic vectors for gene therapy are currently in development and have been shown to safely and efficiently transfect airway epithelial cells in pre-clinical studies [238]. A clinical trial delivering CFTR gene to the lungs and nose using lipoplex vector has been carried out with promising results in patients with cystic fibrosis [237, 239].

Cell based gene therapy. Cell-based therapies with embryonic or adult stem cells have emerged as potential novel approaches for several lung diseases, including ARDS. Although initial studies suggested engraftment of exogenously administered stem cells
in lung, this is now generally felt to be a rare occurrence of uncertain physiologic significance. More recent studies have demonstrated that paracrine effects of stem cells can modulate local inflammatory and immune responses in multiple rodent ALI models. Based on these studies and on safety and initial efficacy data from trials of adult stem cells, many clinical trials of cell-based therapy have been initiated for pulmonary hypertension and for chronic obstructive pulmonary disease[240].

1.6.4 INSIGHTS FROM PRECLINICAL STUDIES

Most of the animal studies investigating the potential of transgene delivery to attenuate ALI were carried out using Adenovirus serotype 5 [Ad5]. These studies have shown that Ad5-transgene pre-treatment protected against *Pseudomonas aeruginosa* pneumonia [241], hyperoxia and ischaemia-reperfusion [242], CLP/LPS induced sepsis and sepsis induced ALI [243, 244], IgG immunocomplex induced ALI [245], and VILI [246]. Genes involved in these studies included, enzymes responsible for surfactant synthesis [241], SOD/catalase [242], angiopoetin-1 [243], apolipoprotein A-1 [244], defensin β2, Na⁺-K⁺-ATPase α₂ [246] and β₁ subunits [223]. Conversely, over-expressing IL-1β [245] or suppressor of cytokine signaling-3 [247] worsened ALI in sepsis and immunocomplex mediated ALI indicating the central role of these cytokines in the pathomechanism of lung injury.

Few preclinical studies have used Ad vector delivery to investigate the role of the NF-κB pathway in ALI/ARDS. Selective transduction of airway epithelial cells by adenoviral vectors [Ad] containing either IKK-α or IKK-β transgenes resulted in constitutive activation of NF-κB [209]. This resulted in increased cytokine release and neutrophil infiltration similar to the pathological changes seen in ALI. Conversely, Ad5 expressing a dominant inhibitor of NF-κB prevented lung inflammation in these animals [211]. In an orthotropic lung transplant model Ad5 associated IκB-α-SR gene delivery attenuated ischaemia-reperfusion injury in rats [213].

In spite of the large number of clinical studies utilising AAV vectors for gene transfer, and increased tissue tropism of AAV5/6/9 serotypes to lung tissue, these vectors have not been used frequently in *in vivo* models of ALI/ARDS. This might due to partially to the larger packaging capacity and easier vector manufacturing of Ad virus vectors
compared to AAVs. Most recently our group reported successful EC-SOD gene transfer using AAV6 serotype to lung epithelium [233]. Hassett et al demonstrated that EC-SOD overexpression enhanced the antioxidant capacity of the lung and attenuated LPS-induced lung injury in rats [233].

1.7 HYPERCAPNIA AND THE NF-κB PATHWAY IN ARDS - KEY UNKNOWNS

ARDS is a devastating disease and constitute a significant burden to society. It has been increasingly recognised that the course of the acute illness has great impact on long term outcome indicating that deeper understanding of the underlying pathomechanism of ARDS and the consequences of therapeutic/supportive approaches in the critically ill is paramount.

Permissive hypercapnia in the context of lung protective mechanical ventilation has been adopted in the management of ALI/ARDS. The question whether this “side effect” is beneficial or harmful still need to be answered. Preclinical studies aiming to investigate how hypercapnia affects the different stages of ARDS, different aetiologies and outcome may be the right approach to obtain more insights.

There are also gaps in our knowledge as to how hypercapnia affects cell functions and what are the potential intracellular mechanisms involved. The potential of hypercapnia to modulate the NF-κB pathway and through this to influence inflammatory and reparative processes may carry significant risks and benefits in critically ill patients.

Despite of enormous amount of experimental and clinical research in ALI/ARDS, there are no specific therapies for ARDS. This is related to the complexity of the disease and consequently, to our incomplete understanding of the diverse pathophysiological processes involved. Advancements of modern gene technologies may help to identify ARDS subgroups, to investigate key pathophysiological processes in ARDS as well as inform devising of new therapeutic approaches.

1.7.1 IS HYPERCAPNIA SAFE IN PROLONGED SYSTEMIC SEPSIS?

A significant amount of work has been carried out to investigate the effects of elevated CO₂ in the acutely injured lung. Initial data suggested that hypercapnia is protective in the early, hyperacute phase of ALI [including sepsis induced ALI], however most recent
results from preclinical data indicate that prolonged hypercapnia may inhibit the immune system with potentially harmful consequences in pulmonary sepsis. The possibility that permissive hypercapnia may worsen systemic sepsis and ALI has significant importance in mechanically ventilated patients. Since sepsis and sepsis induced ALI are the leading causes of death in the critically ill, it is important to investigate the effect of hypercapnia in this setting.

*Based on these, our first experimental objective was to investigate the efficacy and safety of sustained hypercapnia in prolonged systemic sepsis and sepsis induced ALI in an in vivo caecal ligation and puncture [CLP] induced-sepsis model. We wished to determine the effect of prolonged hypercapnia on survival, physiological and inflammatory indices and how these effects relate to the NF-κB pathway.*

1.7.2 WHAT IS THE ROLE OF NF-κB PATHWAY IN MEDIATING THE EFFECTS OF HYPERCAPNIA?

Mechanical ventilation is the most important supportive measure in the management of ARDS. However, it is inherently the source of a subsequent second hit injury termed ventilation induced lung injury. Current research indicates that NF-κB is one of the key molecules mediating the biological effects of VILI. Permissive hypercapnia, in the presence of protective lung ventilation, may have additional modulating effect on the evoked inflammatory response.

There are very few studies on how hypercapnia affects the NF-κB pathway and these are mainly limited to *in vitro* observations. These data suggest that both the beneficial and harmful effects of hypercapnia are mediated via inhibition of the NF-κB pathway. Given the fact that the effects of permissive hypercapnia and mechanical ventilation share common key pathways, such as the NF-κB cascade, it is important to investigate how these supportive measures influence inflammatory processes in ALI/ARDS in order to limit adverse outcomes related to mechanical ventilation.

*Our second experimental objective was to investigate the role of inhibition of the NF-κB pathway in mediating the effects of hypercapnic acidosis. We proposed to evaluate this mechanism in the setting of both sepsis [cecal ligation and puncture] and non-septic [high stretch ventilation] lung injury.*
1.7.3 CAN WE DIRECTLY MODULATE THE NF-KB PATHWAY FOR THERAPEUTIC EFFECT?

Hypercapnic acidosis is a potent biologic agent with diverse effects on the inflammatory and repair response to injury. A key mechanism of action of HCA appears to be mediated via inhibition of the NF-κB transcription pathway. Strategies which directly modulate the NF-κB pathway in the lung epithelium may allow us to harness the benefits of this approach, while limiting the potential for systemic adverse effects. Pulmonary gene therapy offers the potential to realise these objectives in ALI/ARDS and this concept has been supported in diverse preclinical studies. However, the potential of gene therapy has not been investigated in the context of ventilation induced lung injury.

*Given the rationale for gene delivery to ARDS and the role of NF-κB pathway in the pathophysiology of VILI, our third experimental objective was to determine whether adeno-associated viral mediated intrapulmonary gene delivery of an NF-κB inhibitor - IκB-α super-repressor - would modify acute lung injury evoked by excessive mechanical stretch in an in vivo model. We investigated the efficiency of transgene delivery and its impact on lung injury and inflammatory markers.*
CHAPTER 2
CHAPTER 2 : AIMS AND HYPOTHESES

2.1 OVERALL AIM

To investigate the role of the NF-κB pathway in mediating the effects of hypercapnic acidosis in prolonged systemic sepsis and ventilation induced lung injury.

2.2 SPECIFIC AIMS

(a) To determine the safety of hypercapnia in prolonged systemic sepsis and sepsis induced ALI.

(b) To determine the effect of hypercapnia on pulmonary NF-κB pathway activity in prolonged systemic sepsis induced ALI and in ventilation induced lung injury.

(c) To determine the effect of hypercapnic acidosis in ventilation induced lung injury in two VILI models with distinct severity.

(d) To determine the role of NF-κB pathway mediating the effect of hypercapnic acidosis in ventilation induced lung injury.

(e) To directly modulate NF-κB pathway by transpulmonary gene delivery to explore the pathomechanism and the potential for gene therapy in ventilation induced lung injury.

2.3 OVERALL HYPOTHESIS

That the effects of hypercapnic acidosis in prolonged systemic sepsis and ventilation induced lung injury are mediated via inhibition of the NF-κB Pathway.

2.4 SPECIFIC HYPOTHESES

(a) Sustained hypercapnia will worsen systemic sepsis and sepsis induced ALI in vivo.

(b) Prolonged hypercapnia will worsen sepsis induced ALI by inhibiting pulmonary NF-κB pathway activity.

(c) Hypercapnic acidosis will attenuate moderate and severe VILI in vivo.

(d) Hypercapnic acidosis will attenuate VILI by inhibiting pulmonary NF-κB activity.
(e) Transpulmonary delivery of an NF-κB inhibitor – the IκB-α super-repressor – will directly protect against excessive mechanical stretch \textit{in vivo}.
CHAPTER 3
CHAPTER 3: MATERIALS AND METHODS

3.1 ANIMAL CARE

Specific-pathogen-free adult male Sprague-Dawley rats were used in all experiments. All work was approved by the Research Ethics Committee at National University of Ireland Galway, conducted under license from the Department of Health, Ireland and complied with European Union directive 86/609 on the protection of animals used for experimental and other scientific purposes. The animals were housed in individually ventilated cages [Tecniplast®; Buguggiate, Varese, Italy] and allowed water and food ad libitum. Animals were observed three times a day for 96 h during the series where animals were subjected to intervention [CLP, intratracheal viral vector instillation] and received fluid and pain relief in order to reduce distress.

3.2 PROLONGED SEPSIS MODEL - CAECAL LIGATION AND PUNCTURE [CLP]

3.2.1 INTRODUCTION

Sepsis is the most common predisposing condition leading to ARDS [8]. Our group [131] and others [132] have recently demonstrated that HCA reduces lung injury in the setting of early systemic sepsis. However, the safety of HCA in the context of pulmonary sepsis [133] and repair [134] has been questioned. Therefore, we have developed a systemic sepsis model in order to examine the effect of HCA in prolonged systemic sepsis induced lung injury [See Chapter 4]. In this section we discuss various in vivo sepsis models and the rational to choose a CLP model for our experiments.

3.2.2 PERITONITIS MODELS

3.2.2.1 Caecal ligation and puncture model

Caecal ligation and puncture model has been described first by Wichterman et al in 1980 [248], and is one of the most widely used model of sepsis and septic shock [249]. CLP in rodents resembles the clinical situation of bowel perforation leading to systemic inflammation caused by mixed enteric pathogens. Wichterman et al set out the following criteria in order to mimic the pathophysiological events seen in septic patients: (1) polymicrobial sepsis; (2) focal infection in origins; (3) positive blood culture; (4) metabolic and physiological perturbations consistent with systemic sepsis;
(5) easily reproducible model. Recently, Brooks et al have systematically investigated the behavioural and physiological changes that occur during CLP induced sepsis in rats [250]. The group also showed that the most common organism isolated from blood cultures were: *E.Coli, Proteus spp., Bacteroides spp., Enterococcus faecalis, M. Morganii, and α-haemolytic Streptococcus*; demonstrating that CLP resulted in bacteraemia of faecal origin. All CLP animals showed significant reduction in locomotor activity, food intake, body weight, and blood glucose levels. Four and 5h after surgery, animals became pyrexial followed by a gradual decrease of temperature. At the end of the experiment, all CLP animals were hypothermic compared to controls. CLP also resulted in leucopaenia and thrombocytopaenia and increased neutrophil/lymphocyte ratio. Organ dysfunction was assessed by histological analysis demonstrating early signs of tissue damage in the frontal cortex, hepatic and lung tissue.

Hemodynamic response to CLP in rodents is characterized by low arterial blood pressure [BP], increased heart rate [HR], increased cardiac output [CO], stroke volume index [SVI], and decreased cardiac contractility [251, 252]. However, these changes only occur, when aggressive fluid resuscitation is present [251], another important observation supporting the suitability of this model to mimic human sepsis.

The other advantage of the CLP model, that, it is possible to tailor the degree of inflammation to produce full spectrum of severity, ranging from acute fatal to chronic sepsis. Otero-Antón et al have shown that severity of sepsis can be modulated by varying the puncture size in the caecum [253]. The authors found that increasing the puncture size from 22g up to a 0.5 cm blade incision, mortality increased from 27% to 95% and this was associated with increasing plasma endotoxin and TNF-α levels. Other factors, such as distance of caecum ligated, can also determine mortality and severity of sepsis [254]. Addition of fluid resuscitation and appropriate adjuvant supportive treatment, such as antibiotic administration, surgical removal of caecum and washout, makes the CLP model clinically more relevant compared to other sepsis models [249, 250, 255, 256].
3.2.2.2 Colon ascendens stent peritonitis [CASP] model

Colon ascendens stent peritonitis model has been used to induce sepsis in rodents [257]. In this model a stent is inserted into the ascending colon providing constant faecal soiling into the peritoneal cavity leading to severe sepsis. Generally, mortality rate is very high reaching 100% within 48 hours and it is associated with significant organ dysfunction. In contrast with CLP, the macroscopic picture of the peritoneal cavity 24 hours after stent insertion is featured with diffuse faecal peritonitis, massive oedema, paralytic ileus and bowel hyperaemia. Although pathophysiological changes after CASP are similar or somehow more severe to CLP, this model represents a situation of a large anastomotic leak following bowel surgery rather than a bowel perforation induced sepsis [258].

3.2.2.3 Faecal pellet model and bacterial inoculum models

The faecal pellet and bacterial inoculum models have also been studied as sepsis models in rodents. In case of faecal pellet peritonitis faeces is instilled into the peritoneal cavity which can lead to rapid death of the animals. However, it is well known that both the dose and the strain of the bacterias are impossible to quantify, therefore making this model highly variable [248, 258]. The bacterial inoculum model is carried out by implanting an infected fibrin clot into the abdomen containing known amount of bacteria, such as E. Coli. This model is more reproducible and more controlled than the faecal pellet model; however it is associated only with moderate cardiovascular response and represents abscess formation with self limiting inflammation rather than intra-abdominal sepsis resulting in multi organ failure [259].

3.2.2.4 Other sepsis models

Intravenous endotoxin [LPS] or live bacteria into the blood can induce “sepsis-like” states experimentally and are useful to characterize inflammatory response patterns. However, these models have several limitations. (1) High intravenous doses of endotoxin or live bacteria generally produce a rapid decrease in CO and leads to significant mortality within few hours, therefore such an insult may not describe an outcome and progression of the disease that is relevant to human sepsis [248, 260, 261]. (2) The observed hypodynamic circulatory response within short period of time is
very distinctive from the hyperdynamic circulatory response seen in patients with septic shock [248, 259]. Intravenous bolus of endotoxin causes profound decrease in CO, increases systemic vascular resistance [SVR] and decreases SVI both in rats and dogs [259, 262]. (3) Intravenous endotoxin or live bacteria administration causes a transient and much greater cytokine response which may not be typical of those seen during septic states that progress from a focus of infection [263]. (4) Use of one type of bacteria or endotoxin in experimental models is clearly different from patients with infections caused by multiple organisms. The above mentioned observations, therefore, may explain why immunomodulatory agents, such as anti TNF-α monoclonal antibody, anti-LPS antibody or methyprednisolone, resulted to be effective in the treatment of sepsis in animal models using i.v. live bacteria and failed to show any benefit in clinical trials in humans [258, 261, 264]. In contrast with these, endotoxin models using sublethal doses or continuous infusion of low dose endotoxin or intraperitoneal endotoxin administration may generate sepsis more similar to the clinical syndrome [260]. The physiological response evoked by this method is more prolonged and when it is associated with adequate fluid resuscitation it seems to induce a hyperdynamic cardiovascular response [265, 266].

3.2.3 JUSTIFICATION OF CLP MODEL

Based on the above mentioned observations and our pilot data [See Chapter 4] we used a rat CLP model for several reasons:

(1) CLP model closely simulates the human disease process during sepsis.
(2) CLP is reproducible and the model can be tailored to achieve the required severity of sepsis.
(3) Mortality studies in rodents after CLP have shown that animal survival has a certain pattern. During the first 48 hours after CLP, survival decreases in variable degree depending on the puncture size, percentage of caecum ligated, etc.; however after that, survival is stabilized over time [251, 253, 254, 256, 257]. This specific pattern allows us to investigate the effect of HCA in septic animals over a more prolonged time period.
(4) Variability of the model which is generally introduced during the surgical procedure can be easily controlled by performing the surgery at the same time of the day, non-fasting animals, same operator, standardizing operation and anaesthesia and postoperative care.

3.2.4 CAECAL LIGATION AND PUNCTURE PROTOCOL

Adult male Sprague-Dawley rats were anaesthetised with Isoflurane and ketamine 80 mg/kg [Ketalar; Pfizer, Cork, Ireland] intraperitoneally. The lower half of the abdomen was shaved and disinfected with 100% alcohol, and the caecum was mobilized through an approximately 2 cm long, median abdominal incision. The caecum was filled by gently “milking back” colon contents and then ligated at 37.5% of its length with a 3.0 silk ligature distal to the ileo-caecal valve without causing bowel obstruction. The caecum was then subjected to two through and through perforation with a sterile 21-gauge needle and gently compressed until its contents began to exude, to ensure patency of the perforation sites. The bowel was then repositioned, and the abdominal incision was closed in two layers with 4.0 silk sutures. All animals received 20 ml/kg normal saline subcutaneously [s.c] immediately after surgery and allowed to recover from anaesthesia.

3.2.5 RANDOMIZATION AND TREATMENT PROTOCOL

Once animals had recovered from anaesthesia, they were placed in individually ventilated cages and placed under close observation. Each animal was then randomly assigned to be housed under conditions of normocapnia or hypercapnia in an environmental chamber for 96 hours [Figure 3-1]. In animals that were randomized to hypercapnia, ambient oxygen was maintained at 21% and carbon dioxide at 8% using automated controllers [ProOx 110 and ProCO2 120; Biospherix, Lacona, NY]. The use of 8% environmental CO$_2$ resulted in moderate hypercapnia and acidosis which has been piloted previously in our lab by Costello J and Higgins BD [131, 187]. Rats randomized to normocapnia were maintained in 21% oxygen without added CO$_2$ during this time. An opioid analgesic, Buprenorphine [Temgesic®] 0.01-0.05 mg/kg, was administered postoperatively three times a day to provide adequate pain relief based on a distress
scoring system [Table 3-1] [267]. In addition, 30 ml/kg normal saline was administered s.c. every 8h over the following 96 hours.

**Figure 3-1** Schematic overview of CLP protocol. After CLP, Sprague-Dawley rats were randomized and exposed to either hypercapnia [FiCO₂: 0.08] or normocapnia for 96 hours. Subsequently the animals were anaesthetised and ventilated. The time points indicated in the figure represent measurement points, including arterial blood gas analysis, and lung compliance. At the end of the experiment animals were exsanguinated, and blood, peritoneal fluid, bronchoalveolar lavage, and lung tissue were saved for further investigation.
Table 3-1 Distress scoring system was used to assess animal distress post surgery and after vector instillation. Normal: 0-4; Distress: 5-12 [opioid analgesic was administered in these cases and at higher scores euthanasia considered; if the total score was 12 or more, animals were euthanized to prevent suffering.

3.2.6 ANAESTHESIA, DISSECTION AND ASSESSMENT OF INJURY

After 96 hours, anaesthesia was induced with intraperitoneal ketamine 80 mg/kg [Ketalar; Pfizer, Cork, Ireland] and xylazine 8 mg/kg [Xylapan; Vétoquinol, Dublin, Ireland]. After confirming depth of anaesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein, and further anaesthesia was maintained with an intravenous Saffan infusion [Alfaxadone 0.9% and Alfadolone acetate 0.3%; Schering-Plough, Welwyn Garden City, United Kingdom] at 5-20 mg/kg/h. A tracheostomy tube [2-mm internal diameter] was inserted and secured and intra arterial access [22g cannula; Becton Dickinson, Cowley, United Kingdom] was placed in the right carotid artery. After confirmation of depth of anaesthesia by using paw clamp, Cisatracurium besilate [0.5 mg; Nimbex, GlaxoSmithKline, Dublin, Ireland] was administered intravenously to produce muscle relaxation. The animals were
ventilated by using a small animal ventilator [Model 683, Harvard Apparatus, Kent, United Kingdom] with respiratory rate of 90 breaths/min, tidal volume of 6 ml/kg, and PEEP of 2.5 cmH$_2$O. The animals randomized to hypercapnia were ventilated with an inspired gas mixture of 5% CO$_2$, 30% O$_2$, and 65% N$_2$. In the normocapnia group animals were ventilated with an inspired gas mixture of 30% O$_2$ and 70% N$_2$. To minimize lung derecruitment, a recruitment manoeuvre consisting of a PEEP of 10 cmH$_2$O for 25 breaths was applied after initiation of mechanical ventilation and at the end of the 20 min baseline period. Depth of anaesthesia was assessed every 15 minutes by monitoring the cardiovascular response to paw clamp. Body temperature was maintained at 36-37.5°C by using a thermostatically controlled blanket system [Harvard Apparatus, Holliston, MA] and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures, and temperature were continuously measured throughout the experimental protocol. After 20 min mechanical ventilation, an arterial blood gas sample was drawn for blood gas measurement [ABL 705; Radiometer, Copenhagen, Denmark], and lung compliance was measured to obtain baseline measurements. Subsequently, animals were ventilated for 15 minutes, arterial blood gas sample was taken and lung compliance was measured. Finally, animals were ventilated for another 15 minutes on FiO$_2$: 1.0 to determine alveolar-arterial oxygen difference [DAaO$_2$]. At the end of the experiment animals were exsanguinated and blood, peritoneal fluid, bronchoalveolar lavage, and lung tissue were saved to assess injury.

3.3 VENTILATION INDUCED LUNG INJURY MODEL

Ventilation induced acute lung injury model has been well established in the literature. However, development of a model that is suitable to test specific research question may need extensive piloting. In the present work, our aim was to study the effect of HCA in acute VILI and how this effect is linked to the activity of the NF-κB pathway in rat lungs. This section describes our final VILI model and the various factors that may determine the injury pattern.
3.3.1 CHARACTERISTICS OF VILI IN ANIMAL MODELS

The effect of mechanical ventilation in animal models can vary depending on many factors: (1) size of the animal species [small vs. large species]; (2) intensity of injury [i.e. pressure and volume changes during injury] and duration of ventilation [exposure time]; (3) age of the animal. In this section we summarize briefly the literature on factors influencing injury severity and dynamics in VILI in animal models and our final VILI protocols. Our pilot data, in Chapter 6.0, describes the steps of model development in more details.

3.3.2 DYNAMICS OF VENTILATION INDUCED LUNG INJURY IN DIFFERENT SPECIES

Small animal species, such as rats, rabbits and mice [50, 51], are more susceptible to injurious mechanical ventilation than larger animals. *Webb and Tierney* have shown first that rats ventilated with peak inspiratory pressure [PIP] of 14 cmH$_2$O for 1h did not develop significant lung injury, however, 30 cmH$_2$O airway pressures after 1h ventilation resulted in moderate interstitial edema formation. Rats ventilated even higher PIP, 45 cmH$_2$O, for 13-35 minutes developed rapid lung injury with profuse edema and alveolar flooding [51]. This pattern has also been demonstrated by others, and generally it is accepted that in a rat model of ventilation induced lung injury both macroscopic and microscopic changes mainly occur over 30cmH$_2$O of PIP after 1-3h of mechanical ventilation, while over 40 cmH$_2$O of PIP lung injury develops within 30 minutes [113-115].

In contrast with this, larger animals require much longer period of ventilation to achieve the same injury [116-119]. Sheep ventilated with PIP:15 cmH$_2$O or 20 cmH$_2$O for 48h did not develop significant macroscopic injury, and even more aggressive ventilation, using 50 cmH$_2$O of PIP, took around 35 hours to result in sever ALI in some cases [116]. Moderately high peak inspiratory pressures, such as 30 cmH$_2$O, were well tolerated over 48h even with obvious alterations in FRC, compliance, oxygenation, and histological injury scores [117]. Equally, in a porcine model, 50 ml/kg V$_t$ ventilation generating PIP between 40 and 50 cmH$_2$O for 4h did not cause significant macroscopic lung injury [118].

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The observed difference between small and large animal species may be explained by the following factors:

(1) **Anatomical factors.** Mercer et al demonstrated that as the alveolar size and radius increases with larger species, the thickness of interstitial space increases too [268]. Thicker interstitial space was related to the presence of increased stress bearing elements, such as collagen and elastin, in large species [baboons, monkeys, humans]. Others, have shown, that capillary stress failure - a pathophysiological process which contributes in the development of pulmonary edema during injurious ventilation - varies among species. Birks et al have shown in rabbit, dog and horse, that to achieve the same capillary stress failure in the pulmonary circulation, the animals needed increasing transmural pressures; namely 50 in rabbits, 90 in dogs and 130 cmH$_2$O in horses. These differences were related at least in part to thinner blood gas barrier and larger capillary radius in rabbits and dogs compared to horses [269].

(2) **Non linear relationship between lung volumes, alveolar size and body weight.** Based on Mercer and colleague's measurements [268], others suggested, that alveolar size/lung volumes are not linearly related across the various species. Gattinoni et al calculated from these data, that for example the same 10 ml/kg $V_t$ change would cause ~10% increase in alveolar diameter in humans, while this would be much higher in rats, around 25%, and even higher in mouse reaching 40% [270]. As a consequence of this non linear relationship, larger tidal volume ventilation would result in even larger alveolar distension, rendering smaller species more susceptible to VILI.

As a result of differences in susceptibility to VILI, the evolution and the pathological features of injury also varies among small and large species. Pulmonary edema develops very rapidly in small animals leaving shorter time to recruit neutrophils into the lung tissue. In contrast with this, large animals need several hours to develop lung injury, therefore, the pathological picture of VILI is mainly dominated by neutrophil infiltration and inflammation rather than excessive mechanical injury [50].

### 3.3.3 INTENSITY OF LUNG INJURY AND THRESHOLD PHENOMENA

Severity of VILI in response to increasing pressures, volumes or exposure time, changes in a non-linear, most probably an exponential fashion. This assumption implies, that
there is a threshold [pressure, volume, time] when the injury starts to accelerate and leads to severe organ injury. This concept was suggested by Parker et al [119], and summarized in a comprehensive review by Dreyfuss and Saumon[50]. It is important to notice that these parameters largely interrelate therefore it is extremely difficult to investigate their roles independently from each other. It also adds to the complexity that different animal species, for reasons already discussed earlier, express different threshold values. Also, animal batches, age of the animal, experimental conditions [i.e. in vivo vs. ex vivo] also determine the nature of the injury. However, as a good estimate, we can say that manifest lung injury starts to develop over 30 cmH₂O of peak airway pressure, and becomes severe at around 40 cmH₂O PIP [50, 51, 82, 115, 119]. These values are useful starting points to guide in vivo model development and were taken into consideration in our pilot work [See Chapter 6].

3.3.4 AGE OF THE ANIMALS

Age influences susceptibility to ventilation induced acute lung injury. Most preclinical studies suggest that younger animals [neonates, infants] are more capable to withstand injurious ventilation than adults [271, 272]. Aging in adult animals is also associated with less resistance to mechanical stretch [273]. The difference between infantile and adult lung has been investigated more extensively than the effect of aging in adult animals. In general, susceptibility may be related to: (1) changes in mechanical interdependence between airways and parenchyma [274]; (2) changes in chest wall compliance during development and aging [e.g. highly compliant chest wall in young animals may result in smaller distending pressures causing less stress in the alveoli]. (2) changes in collagen and elastin concentrations in the lung [272]. (3) As a result of immature immune system, newborns respond with less inflammation to injurious ventilation than adults [272, 275]. Also, older adults have more pronounced pro-inflammatory response to injury in general than younger adults [276]; (4) total lung volume related to body weight changes during development, being much larger in newborns then in adults, therefore the delivered Vₐ based on ml/kg may represent different strain in newborn animals compared to adults [270, 272].
3.3.5 **JUSTIFICATION OF VILI MODEL**

Based on the above described observations and our pilot data we developed an *in vivo* model of VILI with the following characteristics:

(1) We used a rat model of VILI, since these animals have a well described injury pattern in the literature and the model is reproducible.

(2) In order to reduce variability in response to injurious ventilation, animals were from the same batch and had similar age and body weight in each series.

(3) Since the intensity of mechanical ventilation influences the rate of injury, we performed a series of pilot study to obtain the desired injury [See Chapter 6]. Considering that in small animals VILI can develop very rapidly if the set injury is too strong, we had to balance injury strength [optimal combination of PIP and RR] to exposure time in order to determine a ventilator setting which resulted in reasonable survival, measurable changes in oxygenation, lung compliance, permeability and inflammation over a 4 hour timeframe.

(4) We defined two VILI models to investigate the effect of HCA on the NFκB pathway. *Moderate VILI* was induced by altering the mechanical ventilation settings as follows: PIP: 30 cmH₂O; RR: 15/min; PEEP 0 cmH₂O for a period of 4 hours; *severe VILI* was induced using PIP: 30 cmH₂O; RR: 18/min; PEEP 0 cmH₂O for a period of 4 hours. Analysis of pilot data and the subsequent inferences are described in Chapter 6.

3.3.6 **ANIMAL SURGERY**

Adult male Sprague-Dawley rats were used in these experimental series. Anesthesia was induced with intraperitoneal ketamine 80 mg/kg [Ketalar, Pfizer, Cork, Ireland] and xylazine 8 mg/kg [Xylapan, Vétoquinol, Dublin, Ireland]. After confirming depth of anesthesia by absence of response to paw compression, intravenous access was gained *via* the dorsal penile vein and further anesthesia maintained with an intravenous Saffan® infusion [Alfaxadone 0.9% and alfadadolone acetate 0.3%; Schering-Plough, Welwyn Garden City, UK] at 5-20 mg/kg/hr. A tracheostomy tube [2 mm internal diameter] was inserted and secured and intra-arterial access [22 gauge cannula, Becton Dickinson, Cowley, U.K.] was placed in the right carotid artery [Figure
Following confirmation of depth of anesthesia using paw clamp, Cisatracurium besilate [0.5mg; Nimbex®, GlaxoSmithKline, Dublin, Ireland] was administered intravenously to produce muscle relaxation. The animals were ventilated using a small animal ventilator [Model 683, Harvard Apparatus, Kent, U.K.] with an inspired gas mixture of FiO₂ of 0.3, respiratory rate of 90 breaths/min, tidal volume of 6 ml/kg, and PEEP of 2.5 cmH₂O. To minimize lung derecruitment, a recruitment maneuver consisting of a positive end-expiratory pressure of 10 cmH₂O for 25 breaths was applied. Depth of anesthesia was assessed every 15 minutes by monitoring the cardiovascular response to paw clamp. Body temperature was maintained at 36-37.5°C using a thermostatically controlled blanket system [Harvard Apparatus, MA] and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures and temperature were continuously measured throughout the experimental protocol. After 20 minutes an arterial blood gas sample was drawn for blood gas measurement [ABL 700, Radiometer, Copenhagen, Denmark], and lung compliance measured, as described below, in order to confirm baseline stability.

3.3.6.1 Exclusion and termination criteria

Prior to entry into the experimental protocol, the following baseline values were required: PaO₂ of > 16.0 kPa, HCO₃⁻ of > 20 mmol/l, and temperature of 36.0-37.5°C. Where the criteria were not fulfilled, variables were reassessed after an additional 15 minutes, during which no specific interventions were performed. Failure to meet the criteria at this point mandated exclusion from the protocol. Following entry into the injury protocol, the experiment was terminated if the mean arterial pressure dropped below 50 mmHg.

3.3.6.2 Moderate VILI protocol

Following induction of anesthesia, tracheostomy insertion and placement of arterial and venous access, and confirmation of baseline inclusion criteria, rats were randomized to normocapnia [Normocapnia; FiCO₂: 0.0] or hypercapnic acidosis [HCA; FiCO₂ 0.05]. Moderate VILI was induced by altering the mechanical ventilation settings as follows: PIP: 30 cmH₂O; RR: 15/min; PEEP 0 cmH₂O for a period of 4 hours [Figure 3-2].
3.3.6.3 Severe VILI protocol

Following induction of anesthesia, tracheostomy insertion and placement of arterial and venous access, and confirmation of baseline inclusion criteria, rats were randomized to normocapnia [Normocapnia; FiCO₂: 0.0] or hypercapnic acidosis [HCA; FiCO₂ 0.05]. Severe VILI was induced by altering the mechanical ventilation settings as follows: PIP: 30 cmH₂O; RR: 18/min; PEEP 0 cmH₂O for a period of 4 hours [Figure 3-2].

![Figure 3-2](image-url) Schematic overview of ventilation induced lung injury protocol [VILI]. Sprague-Dawley rats were randomized and exposed to either hypercapnia or normocapnia for 4 hours. During the moderate VILI protocol, the animals were ventilated with peak inspiratory pressure [PIP] 30 cmH₂O, respiratory rate [RR] 15 breaths/min and zero PEEP. Severe VILI was generated by ventilating the animals with PIP: 30 cmH₂O, RR: 18 breaths/ minutes and zero PEEP.

3.4 INTRATRACHEAL VIRAL VECTOR INSTILLATION PROTOCOL

3.4.1 INTRODUCTION

NF-κB pathway is one of the main regulators mediating the inflammatory response during mechanical stretch induced lung injury [95-98]. Selective modification of the NF-κB pathway applying direct gene transfer to the lung epithelium may serve as an adjunctive measure in mechanically ventilated patients with ALI/ARDS. Many preclinical studies successfully demonstrated that intrapulmonary delivery of transgenes inhibiting IκB-α degradation - the rate limiting step of the NF-κB pathway - via adeno viral vectors reduces lung injury [209, 213]. Our team previously demonstrated, that recombinant adeno associated viral vector 6 [AAV6] containing EC-SOD transgene reduced endotoxin induced lung injury in rats [233], which further
supports the relevance of gene therapy in ALI. In our experiments we wished to investigate how overexpression of IκB-α-super-repressor [IκBα-SR] transgene, a potent inhibitor of the NF-κB pathway, would influence the outcome of VILI in rats. We set out a three group experiment. Animals received recombinant AAV6 [rAAV6] containing IκBα-SR transgene or rAAV6 with no transgene [rAAV6-Null] or surfactant [Vehicle] via tracheal route, and subsequently were exposed to VILI. Injury parameters and transgene expression were assessed. In this section, we describe the intratracheal viral vector instillation protocol, animal surgery and VILI protocol. The choice of AAV vector serotype and the production of rAAV6-IκBα-SR are described in more details in Chapter 8 and section 8.2.3.

3.4.2 INTRATRACHEAL VIRAL VECTOR INSTILLATION PROTOCOL

Adult male Sprague-Dawley rats were used in these experiments. Recombinant adeno-associated viral vector 6 containing IκB-α-super-repressor transgene [rAAV6-IκBα-SR] with CMV [cytomegalovirus] promoter sequence, empty vector [rAAV6-Null] or only surfactant [Vehicle] was instilled into the rat lungs under anaesthesia. Each aliquot of frozen virus containing $1 \times 10^{10}$ viral particles was thawed and mixed in 0.5 % PBS albumin to a final volume of 125 μl. This solution was then added to 125 μl of surfactant preparation of porcine lung [Cursurf® 120mg/ml, Trinity-Chiesi Pharmaceuticals Limited, Cheadle Royal Business Park, Highfield, Cheadle, UK] resulting in a total volume of 250 μl. After randomization, each animal was premedicated with 40 mg/kg of ketamine [Ketalar, Pfizer, Cork, Ireland] intraperitoneally and subsequently placed in a sealed transparent box for 2-3 minutes receiving 2 % Isoflurane with 100% oxygen [Ohmeda®Excel 210 anaesthetic machine]. Adequacy of anaesthesia for laryngoscopy was assessed by response to paw clamp. Subsequently the animal was placed on an intubation rack and an intubating stylet was advanced between the vocal cords under direct vision using an adult human otoscope [Welch- Allyn®, Buckinghamshire, UK][Figure 3-3]. A 14 gauge [BD Insyte®] canula was introduced over the stylet through the vocal cords and then the stylet removed. The viral preparation was then administered down the 14 gauge canula using a 1 ml syringe and a further 2 ml of air administered down the canula to ensure that all viral suspension was directed into the rat lungs and out of the canula. After instillation, the
animal was extubated and placed in an individually ventilated recovery cage [Tecniplast®, Buguggiate, Varese, Italy] for 96h to allow maximal transgene expression in the lungs [209, 213, 233].

Figure 3-3 A. Model SAR-830/P Small Animal Ventilator [CWE SAR 830 AP, CWE Inc, Pennsylvania, USA] was used to ventilate the animals with pressure controlled ventilation. B. Sprague-Dawley rat after animal surgery, attached to the ventilator. The trachea is cannulated and a 22g cannula is inserted into the common carotid artery in order to measure arterial blood pressure and to obtain arterial blood gas samples. C. Sprague-Dawley rat is anaesthetised and being intubated using an otoscope. After removing the otoscope, a 14g cannula was fed over the guide wire and the viral vector containing transgene [rAAV6-1xBα-SR], null vector [rAAV6-Null], or only surfactant [Vehicle] was instilled into the lungs. Subsequently, the animal was extubated, let to recover, and placed in an individually ventilated cage for 96 hours.

3.4.3 VILI PROTOCOL 96 HOURS AFTER VIRAL VECTOR INSTILLATION

After pilot studies, the following protocol was used to achieve adequate lung injury in this series. Briefly, after induction of anesthesia, tracheostomy insertion and placement of arterial and venous access, adult male Sprague-Dawley rats were administered FiO₂: 0.3 and mechanically ventilated with the following settings: PIP:
22.5 cmH₂O; RR: 20/min; PEEP 0 cmH₂O for a period of 4 hours. Maintenance of anaesthesia and animal surgery was carried out the same way as it has been described earlier [Section 3.3.6]. Exclusion and termination criteria were similarly to the VILI protocol [3.3.6.1-3.3.6.2].

3.5 MEASUREMENTS, SAMPLING AND ASSAY PROTOCOLS

3.5.1 MEASUREMENT OF PHYSIOLOGIC VARIABLES

Arterial blood pressure, peak airway pressures, and rectal temperature were recorded continuously during the protocol using MP30 BIOPAC Student Lab PRO® system. Assessment of oxygenation, ventilation, and acid-base status was carried out through arterial blood gas analysis [ABL 700, Radiometer, Copenhagen, Denmark] at baseline and subsequently every hour [at 1, 2, 3, 4 hour]. Static inflation lung compliance was measured immediately before a recruitment manoeuvre, ensuring a standardized lung volume history [Figure 3-4]. Incremental 1 ml volume of room air was injected via the tracheostomy tube, and the pressure attained 4s after each injection was measured until a total volume of 5 ml was injected. After 4 hours, at the end of the protocol the inspired gas mixture was changed to FiO₂: 1.0 for fifteen minutes after which time a blood sample was taken for calculation of the alveolar-arterial oxygen gradient using the alveolar gas equation. At the end of the treatment protocol, heparin [400 IU/kg; CP Pharmaceuticals, Wrexham, United Kingdom] was administered intravenously, and animals were killed by exsanguination.

Figure 3-4 A. Representative snapshots of baseline static compliance measurement. The tracheostomy tube was blocked at the end of expiration and the lungs were inflated using air with 1 ml increments up to 5 ml. Total compliance was calculated as the average of the compliance measured at 1 ml and the compliance measured at 5 ml. p1 indicates airway pressure at 1 ml; p5 is airway pressure at 5 ml. B. Representative snapshot of static compliance measurement from a severely injured lung at 4 hours.
3.5.2 TISSUE SAMPLING

Immediately post-mortem, the heart–lung block was dissected from the thorax and the following sampling procedures were performed:

(1) **Determination of lung wet dry weight ratio:** The basal lobe of the right lung was ligated, removed and the weight of this lung segment was measured immediately. Subsequently the tissue was placed in an incubator at 37°C for 48 h and then the dry weight was determined. Ratio of wet and dry lung weight represented lung fluid content [277].

(2) **Bronchoalveolar lavage [BAL] collection:** Total of 15 ml normal saline in 5 ml aliquots were injected through the trachea in order to wash out both lungs. The returned lavage fluid was collected and saved to determine total cell numbers in BAL fluid and differential cell counts. The remaining BAL fluid was centrifuged for 15 min and the supernatant was saved at -80°C.

(3) **Bacterial colony count:** The concentration of bacteria in the BAL fluid, plasma and sero-sanguineous fluid [SSF] from the abdomen was determined by plating serial dilutions on Colombia blood agar plates [L.I.P. Diagnostic Services, Galway]. A range of dilutions for each fluid were carried out as follows: BAL $10^{-4} - 10^{-6}$; plasma $10^{-8} - 10^{-12}$; and SSF: $10^{-12}$. This range of dilutions had been piloted previously and was found to adequately assess bacterial counts for these conditions and samples. A volume of 0.5 ml of each dilution was pipetted onto each plate and the plates were placed in an oven at 37°C and a count of individual colonies was carried out after ~24h.

(4) **Tissue sampling to determine the NF-κB pathway activity:** The right lung was ligated and the lung tissue cut into 2-5 mm pieces and snap frozen immediately. The samples were stored at -80 °C degree for further analysis.

(5) **Tissue preparation for histological analysis:** After the heart-lung block was dissected from the thorax, and BAL collection was performed, the right pulmonary artery was cannulated and a small incision was performed on the tip of the left atrium. Subsequently the pulmonary circulation was perfused with heparinized saline at a constant hydrostatic pressure of 25 cmH₂O until the left atrial effluent was clear of
blood. The left atrium was ligated and paraformaldehyde [4%] was then instilled through the pulmonary artery catheter at a pressure of 62.5 cmH₂O. The left lung was then inflated through the tracheal catheter using paraformaldehyde in phosphate buffered saline [300 mOsmol] at a pressure of 25 cmH₂O. The pulmonary artery, trachea, and the heart was ligated and then removed and the left lung was isolated and stored in paraformaldehyde. The extent of histologic lung damage was determined using quantitative stereological techniques.

3.5.3 BAL TOTAL CELL COUNT

BAL total cell count was calculated from 1000 µl samples immediately after harvesting. Samples were centrifuged at 16 800 g for 10-15 seconds. 750 µl of the supernatant was discarded and the cell pellet was resuspended in the remaining 250 µl supernatant. 20 µl cell suspension was mixed in a separate Eppendorf tube with 20 µl 0.4 % trypan blue [1:1 ratio].

**Calculation:** Total cell count per ml was determined by using Neubauer haemocytometer at 100x magnification. For an accurate determination, the total number of cells overlying in all eight 1 mm² area [n] were counted and then divided by 8 and then divided by the concentrating factor [4] and multiplied by 10⁴.

3.5.4 BAL DIFFERENTIAL CELL COUNT

In order to determine neutrophil count, a separate sample of 1000 µl of BAL was centrifuged at 16 800 g for 10-15 seconds. The sample was concentrated by taking off of 900 µl supernatant and resuspending the cell pellet in the remaining supernatant. After that, 100 µl of cell suspension was aliquoted into the appropriate well of the Cytospin centrifuge [Shandon Cytospin III, Cytocentrifuge]. The cell suspension was then centrifuged at 200 rpm for 2 minutes. Subsequently the produced cell monolayer was stained with Haematoxylin-Eosin and the percentage of neutrophils in the BAL was determined. Absolute neutrophil count was calculated by multiplying the BAL total cell count by the percentage of neutrophils in the BAL.
3.5.5 BAL COLORIMETRIC PROTEIN ASSAY

Bicinchoninic acid [BCA] assay was performed in order to determine BAL protein concentration using diluted samples [dilution factor 5], [Pierce® BCA Protein Assay Kit, Thermo Scientific]. From each BAL sample, 5 μl was placed into a well of a 96 well plate and diluted up to 25 μl with phosphate buffered saline [PBS] solution. In addition, the standard curve was determined by using known amount of bovine serum albumin [BSA] at a working concentration range of 0 - 2000 μg/ml. Duplicate measurements were performed for each sample and standard curve in order to achieve more accuracy. After preparing the Working Reagent [WR] by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B [50:1, Reagent A:B], 200 μl of the WR was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. Subsequently the plate was covered and incubated at 37°C for 30 minutes and then cooled to room temperature. Light absorbance at 562 nm of blank standard replicates and the individual samples were determined by spectrophotometer [PerkElmer, VICTOR™X3 Wallace, Multilabel Plate Reader].

Protein concentration was calculated as follows:

1. The average 562 nm absorbance measurements of the blank standard replicates was subtracted from the 562 nm absorbance measurements of all other individual standard and unknown sample replicates.

2. Standard curve was created by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in μg/ml [Figure 3-5].

3. Subsequently the standard curve was used to determine the protein concentration of each BAL sample.
Figure 3-5 A representative graph of a standard curve for BCA protein assay. Serial dilution of known concentration of bovine serum albumin [BSA] was used to calculate BAL protein concentration. Y axis: BSA concentration in μg/ml; x axis: Absorbance [Abs] at 562 nm.

3.5.6 BAL CYTOKINE ELISA

Enzyme linked immunoassay kits from R&D Systems Europe Ltd, Abingdon Science Park, Abingdon, OX14 3NB United Kingdom was used to determine BAL cytokine content. 96 well flat bottomed ELISA plates were first coated with the binding antibody and coating buffer [PBS] at 100 µl per well, tightly sealed with parafilm and left to incubate at 4°C overnight. Plates were then subjected to two 180 µl per well washes with PBS Tween [10mM sodium phosphate, 0.15M NaCl, 0.05% Tween-20, pH 7.5]. Blocking buffer of 1% BSA [bovine serum albumin] in PBS 100 µl was added to each well and left for one hour at room temperature. A duplicate standard curve set of samples was constructed with 400 µl neat IL-6, CINC1, TNF-α at the top end of the range, and 7 serial half dilutions of this then made with 1% BSA in PBS as the diluents. The plates were again subjected to three 180 µl per well washes with PBS Tween. Samples were then added in duplicate. Accuracy was tested with pilot experiments and therefore neat serum was used for TNF-α and IL-6 detection, and diluted samples for CINC-1. A total volume of 100 µl [neat or diluted samples with 1% BSA in PBS] of sample was placed in each well. The standard curve samples were added at this stage also at 100 µl per well. A 1 in 400 dilution of detection antibody was added to each well and left to incubate for 2 hours. The plate underwent three 180 µl per well washes with PBS Tween. Streptavidin-HRP conjugate solution [100 µl] was added to
each well and incubated for 20 minutes. The plate was again subjected to three 180 µl per well washes with PBS Tween. Substrate solution [100 µl] was added to each well and the plate left in the dark for 20 minutes. Finally 50 µl of stop solution [2N H₂SO₄] was added to each well and the plate was read at the Wallace plate reader [PerkElmer, VICTOR™ X3 Wallace, Multilabel Plate Reader].

**Optical density was determined as follows:**

(1) Absorbance readings were set at 450 nm and 570 nm.

(2) In order to correct for optical imperfections in the plate, readings from 570 nm were subtracted from the readings from 450 nm.

(3) Standard curve was created by plotting the corrected measurements for each standard vs. its concentration in pg/ml [Figure 3-6].

(4) Subsequently the standard curve was used to determine the cytokine concentration of each serum sample.

![Figure 3-6](image)

**Figure 3-6** Representative graph of a standard curve for ELISA cytokine assay. Serial dilution of known concentration of bovine serum albumin [BSA] was used to calculate in this case BAL CINC-1 protein concentration. Y axis: BSA concentration in pg/ml; x axis: Normalised absorbance.

### 3.5.7 TOTAL PROTEIN EXTRACT FROM LUNG TISSUE

CelLytic™ MT mammalian tissue lysis/extraction reagent [Sigma, SL, US] was used to extract total protein from rat lung tissue samples. For this procedure a ratio of tissue
to CellLytic™ reagent of 1:20 [100 mg tissue: 2 ml reagent] was used. Protease inhibitor was added to the reagent prior to the procedure. Briefly, lung tissue was defrosted and washed with PBS. Each step of the extraction process was performed on ice to prevent protein degradation. The samples and the reagent was transferred into a pre chilled 5 ml tube and homogenized. Subsequently the lysate was centrifuged for 10 minutes at 16 800g at 4°C to pellet tissue debris. The protein containing supernatant was transferred to a chilled test tube. Protein concentration was determined using BCA protein assay kit [Pierce® BCA Protein Assay Kit, Thermo Scientific].

3.5.8 PREPARATION OF CYTOPLASMIC AND NUCLEAR EXTRACT

NE-PER Nuclear and Cytoplasmic Extraction Kit [Thermo Scientific] was used to extract proteins from nuclear and cytoplasmic fractions of lung tissue.

(1) Reagent preparation: Protease inhibitor was added to Cytoplasmic Extraction Reagent I [CER I] in order to maintain extract integrity and function. All steps were performed on ice.

(2) Tissue preparation: 100 mg of defrosted lung tissue was cut into small pieces and placed in a microcentrifuge tube. Samples were washed with PBS and centrifuged at 500g for 5 minutes at 4°C. After removing the supernatant the tissue was homogenized with 1000 μl of CER I.

(3) Cytoplasmic and nuclear protein extraction: Subsequently the homogenized tissue was vortexed vigorously for 15 seconds and incubated for 10 minutes on ice. After that, 88 μl ice–cold CER II reagent was added to the sample and vortexed for 5 seconds followed by 1 minute incubation. The mixture then was centrifuged again at 16 000g for 5 minutes. The supernatant, containing cytoplasmic protein, was transferred into a pre chilled tube and stored at -80 °C until use. The pellet fraction, containing nuclei was then suspended in the Nuclear Extract Reagent [NER], vortexed for 15 seconds every 10 minutes for a total of 40 minutes. The samples were kept on ice during the 10 minutes incubation times. After centrifugation [16,000g for 10 minutes], the supernatant, containing the nuclear protein fraction was transferred to a pre-chilled tube and stored at -80 °C.
3.5.9 ANALYSIS OF THE ACTIVITY OF THE NF-κB PATHWAY IN LUNG TISSUE

3.5.9.1 Western blot analysis of lung tissue IκB-α content

Total cell protein was extracted from thawed, homogenized lung tissue samples using the CellLytic™ MT lysis reagent [Sigma-Aldrich Ireland Ltd. Dublin, Ireland]. Cytoplasmic protein extraction was extracted from thawed, homogenized lung tissue samples using the NE-PER Nuclear and Cytoplasmic Extraction Kit [Thermo Scientific]. Supernatant protein concentration was determined using BCA protein assay kit [Pierce® BCA Protein Assay Kit, Thermo Scientific]. 20 μg from the cytoplasmic, and 30μg of the total protein extract from each sample was loaded on a polyacrylamide gel [Precise™ Protein Gel, Pierce Biotechnology, USA] and electrophoresed in Tris-HEPES-SDS running buffer. Non-specific binding sites were blocked overnight in PBS/non-fat dry milk solution [5% w/v] at 4°C. Primary mouse IκB-α antibody [Cell Signaling Technology] at a dilution of 1:2000 in blocking solution was applied for 12 hours followed by washing the blot paper with Tween 20/PBS [0.05% v/v]. Subsequently the membrane was incubated with anti-rabbit antibody conjugated to horseradish peroxidase [Cell Signaling Technology] for 1 hour at a dilution of 1:2000 in blocking solution. After a second set of washes the membrane was incubated with a chemiluminescent substrate [SuperSignal® West Pico, Thermo Scientific] for 5 minutes and then visualized with Kodak Image Station 4000MM Pro [Carestream Health, Inc., Rochester, N.Y]. IκB-α protein was detected at size 35 kDa. As an internal control each blot was stripped and tested for β-actin content by incubating the membrane with mouse β-actin-HRP antibody [1:20,000] for 2 hours in blocking solution. Densitometry was performed for both proteins and normalized IκB-α levels calculated.

3.5.9.2 Determination of p65 content in nuclear extracts by ELISA

Nuclear protein was extracted from thawed, homogenized lung tissue samples using NE-PER Nuclear and Cytoplasmic Extraction Kit [Thermo Scientific] [Section 3.5.8.] from animals ventilated with injurious ventilation under normocapnia or HCA. Nuclear protein concentration was determined using BCA protein assay kit [Pierce® BCA Protein Assay Kit, Thermo Scientific] and samples were normalized to the same concentration [40 μg/120 μl]. CST’s PathScan® Total NF-kB p65 Sandwich ELISA Kit
[Cell Signaling Technology, Beverly, MA, US] was used to detect endogenous nuclear p65 protein. The microwells were pre-coated with NF-κB p65 antibody by the manufacturer. After the microwells reached room temperature, 100 μl of the normalised sample was added to each well. The samples were tightly sealed with a tape and were incubated for 2 hours at 37°C. Subsequently the wells were washed 4 times with 200 μl 1xWash Buffer provided by the manufacturer. 100 μl of detection antibody was added to each well and left to incubate for 1 hour at 37°C. The plate underwent four 200 μl per well washes with 1xWash Buffer. HRP conjugated secondary antibody [anti-mouse IgG] solution [100 μl] was added to each well and incubated for 30 minutes at 37°C. The plate was again subjected to four 200 μl per well washes with 1xWash Buffer. TMB substrate [tetramethylbenzidine] solution [100 μl] was added to each well and the plate left in the dark for 30 minutes at room temperature. Finally, 100 μl of stop solution [2N H₂SO₄] was added to each well and the plate was read at the Wallace plate reader [PerkElmer, VICTOR™ X3 Wallace, Multilabel Plate Reader]. Optical density measured by light absorbance was determined at 450nm within 30 minutes after adding the stop solution. Nuclear p65 absorbance fold changes were compared between normocapnia and HCA.

3.5.9.3 Determination of nuclear p65 binding activity by ELISA

NF-κB [p65] transcription factor ELISA assay is a sensitive method for detecting DNA binding activity in nuclear extracts and whole cell lysates [Cayman Chemical Company, Mitchigan, U.S.; #10007889]. A specific double stranded DNA sequence containing the NF-κB response element [κB] is immobilized to the wells of a 96 well plate. P65 in a nuclear extract, binds specifically to the κB response element and then detected by addition of specific primary antibody directed against p65. A secondary antibody conjugated with HRP is added to provide a colorimetric readout at 450 nm. This is a sensitive alternative method to the radioactive electrophoretic mobility shift assay [EMSA].

Nuclear protein was extracted from thawed, homogenized lung tissue samples using NE-PER Nuclear and Cytoplasmic Extraction Kit [Thermo Scientific] [Section 3.5.8.] from septic animals exposed to normocapnia or HCA. Nuclear protein concentration was
determined using BCA protein assay kit [Pierce® BCA Protein Assay Kit, Thermo Scientific] and samples were normalized to the same concentration [100µg/100µl]. After the microwells reached room temperature, 10 µl of the normalised sample and 90 µl of the complete transcription factor binding assay buffer was added to each well. The samples were tightly sealed with a tape and were incubated overnight at 4°C. Blank, positive control, non-specific binding wells and competitor dsDNA wells were included in the measurement. Subsequently the wells were washed 5 times with 200 µl 1xWash Buffer provided by the manufacturer. P65 primary antibody was diluted [1:100] and 100 µl of the diluents was added to each well and left to incubate for 1 hour at room temperature. The plate underwent five 200 µl per well washes with 1xWash Buffer. A 1 in a 100 dilution of HRP conjugated secondary antibody [anti-rabbit IgG] solution [100 µl] was added to each well and incubated for another 1 hour at room temperature. The plate was again subjected to five 200 µl per well washes with 1xWash Buffer. Transcription factor developing solution [100 µl] was added to each well and the plate left in the dark exposed to gentle agitation for 15-45 minutes at room temperature. Stop solution, 100 µl/well, was added when the media turned into dark blue colour. The plate was read at the Wallace plate reader [PerkElmer, VICTOR™ X3 Wallace, Multilabel Plate Reader]. Optical density measured by light absorbance was determined at 450nm within 5 minutes after adding the stop solution. Nuclear p65 absorbance fold changes were compared between normocapnia and HCA.

3.5.10 TISSUE PREPARATION FOR HISTOLOGICAL ANALYSIS

(1) Tissue processing and embedding: After fixation, the vertical axis of the left lung was identified. The lung was cut into seven equal sections perpendicular to this axis using a sharp blade [order from apex to base: A, B, C, D, E, F]. After sectioning the fixed lung, the sections were placed in an appropriately labelled histoprocessing cassette [Cat # M490-4, Histosette I, Simport Industries, 2588 Bernard-Pilon, Beloeil QC, J3CG 4S5, Canada]. Also included in the cassette was a pencil inscribed label detailing the animal and section number. The sections were then embedded in paraffin wax using a histoprocessor [ASP 300 histoprocessor, Leica Microsystems, Wetzlar, Germany] applying the factory preset “routine overnight” program. The following morning the cassettes were removed from the processor. Each section was orientated in a
transverse manner, with its top as the cutting edge. Sections were cut at 7 μm using a microtome [Ergostar HM200, Microm Laborgerate GmbH, D69190 Walldorf, Germany] and placed on labelled glass slides. The slides were dried overnight at 37°C to facilitate adherence of the sections to the slides.

(2) Haematoxylin and Eosin staining. The following morning the slides were heated at 60°C for 20 minutes in order to melt the wax. They were then put through two baths of xylene for 10 and 5 min, respectively. [Cat # 305756G, VWR International Limited, Poole, BH15 ITD, England] and graded alcohols [Cat # 1.00983.2500, Merck KGaA, 64271, Darmstadt, Germany] [100%, 95% and 70%, respectively] for 3-3-3 min to rehydrate the tissue. The slides were then placed in a bath of Harris haematoxylin for 6 minutes [# HT110232, Sigma-Aldrich Ireland Ltd. Dublin, Ireland] and subsequently dipped in running tap water for 5 minutes. The slides were placed in acid alcohol [1%] for 10 sec and soaked in tap water for 3 minutes. Samples were then dehydrated in 100% alcohol, 3 times, and two baths of xylene. Finally, samples were covered with a glass cover and mounted in DPX [Cat # 360294H, VWR International Limited, Poole, BH15 ITD, England]. Slides prepared in this manner were stored at room temperature, and protected from light.

3.5.11 STEREOLOGICAL AIRSPACE ANALYSIS

Slides prepared as described above were viewed at a 10X magnification under a microscope [Model BX51, Olympus, Mason Technologies, Dublin 8, Ireland]. Two fields of view from each slide were chosen at random and digitised using a digital camera [Olympus DP70, Mason technologies, Dublin 8, Ireland]. Images were stored in eight-bit [256 level] format. The grid reference, i.e. X and Y grid coordinates, for each image was recorded by referencing the scale attached to the microscope side. A 100 point counting grid was overlaid on each image in AnalySIS® imaging software package [Version 1.20, Olympus, Soft Imaging System, Münster, Germany]. Care was taken to ensure that the software was set to 10X magnification. Once this grid was superimposed over the image, a touch count was performed. At each of 100 intersection points on the grid, a record was taken for each of the following: acinar tissue, non-acinar tissue and airspace. Acinar volume fraction represented all alveolar
tissue including epithelium, endothelium, connective tissue and inflammatory cells. Non-acinar fraction meant bronchial or vascular space, and air space described the gaseous component of the lung. The totals were recorded and analysed using Microsoft Excel™ software.

3.6 GENERATION OF ADENO-ASSOCIATED VIRAL VECTOR

3.6.1 INTRODUCTION

Gene based therapy involves the insertion of genetical materials to cells in order to replace a defective gene or alter the cells to secrete specific proteins into the extra cellular space [218]. ARDS may be suitable for gene therapy for the following reasons: transient nature of the disease requires short term gene-expression, the lung epithelium/endothelium can be accessed from the circulation and via the trachea, targeting different phases of the injury can provide more specific treatments.

Current gene based approaches can be classified depending on the delivery system that is applied to transfer specific genes. In general, vectors can belong to: (1) Viral vector based systems; (2) non-viral gene delivery systems; (3) cell mediated delivery systems. Each approach has its pros and cons; therefore, these details are important and should be considered when a vector system is elected for gene therapy [218]. These factors are summarized in Table 3-2. Ideally, vectors should be safe [non toxic, non pathogenic, non carcinogenic], easy to manufacture and scale up. Long term and stable gene expression, excellent tissue tropism, and feasible manufacturing are also important requirements for an ideal gene vector.

We chose an adeno-associated viral vector system to deliver IκBα-SR transgene [an inhibitory mutant of the IκB-α protein complex] to investigate the effect of NF-κB pathway inhibition in the context of mechanical lung injury. In the present section we describe the manufacturing of a recombinant AAV6-transgene vector construct. We discuss in more details the advantages and disadvantages of AAVs and the rational for using serotype 6 AAV for transgene delivery in Chapter 8.
<table>
<thead>
<tr>
<th>Delivery systems</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td><strong>Viral vectors</strong></td>
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<tr>
<td>Adenoviral vectors</td>
<td>• Easy to produce</td>
<td>• Immunogenic</td>
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<td></td>
<td>• Can deliver large transgene</td>
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<td></td>
<td>• Tolerated in lower doses</td>
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<tr>
<td>AAV vectors</td>
<td>• Safe, non pathogenic</td>
<td>• Limited packaging capacity</td>
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<tr>
<td></td>
<td>• Replication deficient</td>
<td>• Difficult to produce large quantities</td>
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<td></td>
<td>• Transduce both dividing and non dividing cells</td>
<td>• Moderately immunogenic</td>
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<tr>
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<td>• Stable gene expression, stable integration into host genome</td>
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<td>• Have been used in clinical trials</td>
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<tr>
<td>Lentivirus vectors</td>
<td>• Transduce dividing cells</td>
<td>• Oncogenic due to random integration</td>
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<td></td>
<td>• Integrate stable but randomly into host genome</td>
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<td><strong>Non viral vectors</strong></td>
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<td>Closed dsDNAs</td>
<td>• These are plasmids and easy to produce at low costs</td>
<td>• Non specific cell targeting</td>
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<tr>
<td>Lipoplexes/Polyplexes</td>
<td>• Lipid complexes protecting DNA</td>
<td>• Less efficient than viral vectors</td>
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<tr>
<td>DNA/RNA Oligonucleotides</td>
<td>• Easy to produce</td>
<td>• Non specific cell targeting</td>
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<td>• Small molecules, better cellular uptake</td>
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<tr>
<td>Cell based vectors</td>
<td>• Can regulate specific genes</td>
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<tr>
<td>Mesenchymal/stromal stem cell</td>
<td>• Both systemic and tissue specific delivery</td>
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<td>• Can transform into tissue specific cells</td>
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<td>• Have been used in clinical trials</td>
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<td>Fibroblasts</td>
<td>• Same as stem cells</td>
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<td>• Systemic delivery</td>
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**Table 3-2** Summary of vector delivery systems delineating their advantages and disadvantages in relation to production, safety, efficiency and usage in clinical trials [218].

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3.6.2 PRINCIPLES OF VECTOR PRODUCTION AND PURIFICATION

Traditionally, AAV based gene transfer incorporated multiple plasmids decoding AAV sequences carrying either the transgene in question, and gene sequences decoding enzymes that are essential for virus synthesis [Rep genes] linked with genes responsible for capsid synthesis [Cap genes] [220, 278]. However, AAVs are incapable to multiply in cells without helper viruses, therefore co-administration of low dose adeno virus [Ad] provided the additional helper genes for synthesis, packaging and virus release. This system is called the “three-plasmid system” [279, 280]. The main drawbacks of this system is its complexity [requiring multiple plasmids, slower production process, lower viral yield or higher percentage of defective viral vectors, and difficulty to scale up to generate large viral stocks] and the fact that the administered Ad can be harmful to host cells. Over the last two decades many vector transfer system has been tested and continues to be developed. The most common and systemically tested delivery system in AAV vector production consists of two plasmids [“two-plasmid system”], and has been developed by Grimm et al [279]. Administration of AAV plasmids incorporating the transgene in question and co-transfection of a recombinant plasmid containing Rep, Cap segments, and genes solely responsible for virus synthesis from the adenoviral genome, resulted in a more efficient, simpler and safer gene delivery option. The adaptation and the modified version of the two-plasmid system has been successfully used previously in our laboratory as well [description of our transfection method is described in Chapter 8 in more details].

One of the crucial steps of AAV production is the purification process. This step largely influences both the quality and the quantity of the viral stock and can determine experimental outcomes. Zolothukin et al have developed a novel protocol in 1999 for AAV2 purification using Iodixanol gradient ultracentrifugation followed by ion exchange or heparin affinity chromatography [we are referring to more detailed description in Chaper 8] [281]. This method has been proven to be more efficient than the conventional cesium based chromatography. The authors achieved shorter manufacturing time, higher virus titre, and better virus particle-to-infectivity ratio. Since then, this protocol, although with some modifications, has been used to purify
AAV1, 2, 4, 5, 6 and 8 to generate high scale virus stocks for *in vivo* studies [282, 283]. However, Wu *et al* pointed out, that the methodology involved in virus purification is not universal; therefore, purification protocols for each AAV serotype should be individually optimized. Our laboratory has repeatedly used and optimized the above mentioned method to generate recombinant AAV5 and 6 vectors carrying either EC-SOD or IκBα-SR transgene.

3.6.3 SUMMARY OF PRODUCTION STEPS TO GENERATE RECOMBINANT AAV6 VECTOR

Overview of steps of manufacturing recombinant AAV6 generations involved in this thesis [Some of the steps are also presented in Chapter 8 with more detailed explanation. This will be indicated in the text]:

(1) Bacterial transformation.

(2) Generating large stock of plasmid DNA for transfection.

(3) Transfection of 293T [human embryonic kidney cells] with transgene containing plasmid and Rep/Cap plasmid to generate rAAV6-IκBα-SR transgene.

(4) Harvesting transfected 293T cells.

(5) Preparation of vector containing crude cell lysate.

(6) Iodixanol gradient ultracentrifugation.

(7) Ion exchange chromatography.

(8) Desalting and concentration of viral containing eluent.

(9) Real time-PCR to determine virus titre.

3.6.3.1 Bacterial transformation

We used One Shot®TOP10F competent *E. Coli* cells [Invitrogen, CN: #C3030-03]. For each plasmid, 50 μl of competent *E. Coli* suspension [1 vial] was thawed from -80°C freezer on ice. 1-5 μl of plasmid stock [pAAV-IκBα-SR, pRep/Cap6] was then added to the thawed cell suspension and tapped gently to ensure thorough mixing. The mixture
was incubated on ice for 30 minutes and subsequently exposed to heat shock for 30 seconds in a 42°C water bath. Following the heat shock treatment, the cell suspension was placed on ice for 2 minutes before addition of 250 μl pre-warmed [room temperature] SOC medium [Super Optimal Broth with catabolite repression] to each vial. The vials were then placed in a rotary-shaker incubator for 1 hour at 225 rpm. 50 μl of the cell suspension was aseptically spread on ampicillin supplemented [100μg/ml] Luria Berani [LB] agar [1% w/v] plate. The plates were then incubated at 37°C for 18 hours in an inverted position [to avoid condensation].

3.6.3.2 Giga preparation of plasmid DNA

The essence of this step was to generate large amount of plasmids that would be sufficient for transfection. The purification protocol was based on a modified alkaline lysis procedure [precipitation], followed by binding the plasmid DNA to an anion-exchange resin [ion exchange resin purification]. RNAs, proteins, dyes and other impurities were removed with low salt wash and the plasmid was eluted in a high salt buffer. Subsequently the plasmid containing eluent was concentrated and then desalted with isopropanol.

Briefly, a starter bacterial culture was generated by picking bacterias containing either pRep/Cap6 or pAAV-IκBα-SR. A single *E. Coli* bacterial colony was placed into 5-10 ml LB medium containing ampicillin [100 μg/ml] and was incubated for 8 hours on a rotary shaker at 300 rpm at 37°C. The starter culture subsequently was added to 2.5 l LB broth containing 0.25 g ampicillin to produce more bacteria. The mix was incubated for 12-16 hours and placed on a shaker at 300 rpm at 37°C. The bacterial culture was then centrifuged at 6000g for 15 minutes at 4°C.

Supernatant was discarded and the bacterial pellet resuspended in 125 ml Buffer P1 [50 mM Tris- HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A]. The cell suspension was vortexed and pipetted up and down to make sure that the pellet homogenously resuspended. Subsequently 125 ml Buffer P2 [200mM NaOH, 1% SDS (w/v)] was added and vigorously mixed through 4-6 times and incubated at room temperature for 5 minutes. 125 ml chilled Buffer P3 [3.0 M CH₃CO₂K, pH 5.0] was then added to the mixture and again vigorously mixed. These phases resulted in the precipitation of the
genomic DNA, proteins and cell debris. The following centrifugation step [20 000 g for 30 minutes at 4 °C] separated the supernatant containing DNA plasmid from the cell pellet containing the rest of the cell components. Equilibration of resin chromatograph [QIAGEN-tip 1000] was carried out using 75 ml Buffer QBT. The buffer was allowed to flow through the column and after that the DNA containing supernatant was loaded on QIAGEN-tip 1000. Once the sample has flown through the resin column and the plasmids adhered to the surface, the QIAGEN-tip 1000 was washed with 600 ml Buffer QC to remove impurities. Plasmid DNA was eluted with 100 ml Buffer QF [1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15 % isopropanol]. DNA was then precipitated with total volume of 70 ml isopropanol and centrifuged at 15 000g for 30 min at 4°C. After removal of the supernatant, the pellet was washed with 10 ml 70% ethanol and centrifuged again [15 000g for 15 minutes at 4°C]. The DNA pellet was air dried and stored at -20°C until yield determination was performed with spectrophotometry.

3.6.3.3 Quantification of plasmid DNA

Plasmid DNA pellet was resuspended in 250-500 μl dH₂O. Quantification of the plasmid DNA was determined by the use of a NanoDrop™ 1000 spectrophotometer. Blank readings at optical density [OD] 260 nm were taken using 2 μl dH₂O. A total of 3 readings per plasmid DNA sample were taken and the average of those measurements calculated. The concentration of the plasmid DNA was expressed as micrograms per milliliter [μg/ml].

3.6.3.4 Transfection of 293T cells

Transfection of 293T cells with pAAV-IκBα-SR and pRep/Cap6 and optimization of the method are described in more details in Chapter 8. In this section we are going to discuss the basic cell culture procedures carried out to generate large cell stock for vector production. All cell culture procedures were performed in HERAsafe Heraeus Class 2 Biosafety cabinet [CSC Laboratory Equipment] under sterile conditions. 293-T cells were cultured at 37°C in T-175 cell culture flasks in DMEM medium [Dulbecco’s Modified Eagle Medium] with 4500 mg/l glucose and supplemented with 10% [v/v] FBS [Fetal bovine serum], 1 % [v/v] L-glutamine and 1 % [v/v] penicillin-streptomycin under a humidified atmosphere of 5% CO₂ and 95 % HEPA filtered air. The culture medium
was renewed every 2-3 days. Cell passage and splitting was performed in order to grow sufficient amount of cells for transfection. We used four confluent T-175 flasks, each flask was sufficient to seed ten 150 mm dishes. Total of 40 dishes were used for transfection.

3.6.3.5 Cell passaging and splitting

293T cells were seed in T-175 cell culture flasks in DMEM [Dulbecoo’s Modified Eagle Medium] medium supplemented with 10 % (v/v) FBS [fetal bovine serum], 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO₂ and 95% HEPA filtered air. The culture medium was renewed every 2-3 days subsequently. Cells were split or passaged [depending on the requirement] to generate sufficient amount of flasks for transfection [4 flasks with confluent cells was sufficient to plate forty 15 mm cell culture dish]. Once cells were confluent, the medium was aspirated and the cells washed with sterile PBS. Following aspiration of PBS, 5 ml 0.25 % Trypsin was added to the flask and placed back into the incubator for 5 minutes. As a result of this, cells become detached from the bottom of the flask. Subsequently, 5 ml medium was added to the suspension to neutralise the effect of trypsin. The cell suspensions were then collected in a 15 ml tube [4 x 15 ml tubes] and centrifuged at 2000g for 5 minutes. Subsequently the supernatant was removed and the cell pellet resuspended in 10 ml fresh 10% DMEM, and 10 μl of aliquot was taken for cell counting. 4 x 10⁶/ml cells were subsequently seeded in 1x 15 mm plate and 60-70% confluence was achieved by 48 hours.

3.6.3.6 Cell plating on 15 mm culture dishes prior to transfection

Total of forty 15 mm dish was prepared and each filled with 19 ml DMEM medium supplemented with 10 % (v/v) FBS [fetal bovine serum], 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin. 1 ml cell suspension containing ~ 4 x 10⁶ 293T cells were added to each dish [total of 1.6 x 10⁸ cells/40 dishes]. The plates were then placed into the incubator at 37°C under a humidified atmosphere of 5% CO₂ and 95% HEPA filtered air for 48 hours. Cells were checked every day for contamination and growing. In general, the cell culture become ~ 60% confluent after 48 hours and was ready for transfection.
3.6.3.7 Transfection

For each transfection, a 1:1 molar ratio of the pAAV6Rap/Cap and plasmid coding IκB-α-SR transgene [pAAV-IκB-α-SR] were combined for a total of 1000 μg of plasmid DNA per forty plates [250μg/10 plates]. The relative molecular weight of each plasmid determined the optimal ratio of which the 1000 μg of total DNA plasmid were divided [See calculations in Chapter 8]. We used 830 μg pRep/Cap6 and 170 μg pAAV-IκBα-SR in order to transfect 40 dishes.

The final optimised transfection protocol is described in this section [for optimization see Chapter 8]. Forty-forty ml sterile 150mM NaCl was placed into two 50 ml falcon tube each. 830 μg pRep/Cap6 and 170 μg pAAV-IκBα-SR was added one of the tube and 2000 μl jetPEI was added to the other tube. Both mix were vortexed gently and then the mix containing jetPEI was added to the plasmid containing mix [of note, mixing the solutions in a reverse order could reduce transfection efficiency]. Subsequently, the solution was vortexed and incubated for 30 minutes at room temperature. 2 ml of the transfection was added into each 15 mm dish [total 80 ml/ 40 dish = 2ml/dish]. We incubated the cells for 4 hours with the transfection mix, and subsequently the agent was removed and replaced with fresh culture medium. After 48 hours transfection efficiency was checked by looking at cell fluorescence under fluorescent microscope.

3.6.3.8 Harvesting cells

After 48 hours of incubation the culture medium was removed from all plates leaving 3-5 ml medium in the plate and the cell monolayer was harvested by scraping off cells from 10 plates and collecting into a 50 ml tube [from 40 plates, 4 x 50 ml tubes]. The cells and the remaining small amount of medium were centrifuged at 500g for 10 minutes. Subsequently the supernatant was aspirated and the cell pellet resuspended in 15 ml of sterile cell Lysis buffer [150 mM NaCl, 50 mM Tris-HCl, pH: 8.5]. In order to break up the cell membranes, the samples were then exposed to freeze-thaw cycle 3 times for 10 minutes alternating in dry ethanol bath and 37°C water bath. Nucleic acids and DNA fragments in the cell lysate were digested by adding 50 U/ml [750 U/15 ml] Benzonase [Sigma Aldrich, Ireland] to the lysate, then vortexed briefly and incubated
for 60 minutes at 37°C. Following incubation, the crude virus containing lysate was centrifuged at 4000g for 20 minutes and the vector containing supernatant was either stored for short term at -80°C or transferred to an OptiSeal™ Ultracentrifuge tube [Beckman Coulter, Ireland] for further purification. Iodixanol gradient centrifugation

This section is explained in more details in Chapter 8 in Section 8.3.11.

3.6.3.9 Iodixanol gradient centrifugation

This section is explained in more details in Chapter 8 in Section 8.3.11.

3.6.3.10 Ion exchange chromatography

HiTrap™ Q-Sepharose HP columns [5ml, GE Healthcare, Sweden, Catalogue number:#17-1154-01] were used with a subsequent protocol for purification [Table 3-3].

(1) **Column equilibration.** The column is washed with equilibration buffers with specific pH and ionic strength to allow optimal environment for rAAV to bind to the chromatography medium and to free the sample from impurities. We used 25 ml of Buffer A [20mM Tris, 15mM NaCl, pH: 8.5] and 25 ml Buffer B [elution buffer: 20ml Tris, 500mM NaCl, pH: 8.5] and again 25 ml Buffer A. Rate of washing was 5 ml/min [Table 3-3, Steps 1-3].

(2) **Binding phase.** 3 ml of the rAAV fraction was mixed with 3 ml Buffer A [1:1 mix] and was passed through the column twice to improve virus binding. Subsequently, the column was washed with 25 ml Buffer A [Table3-3, Steps 4-5].

(3) **Elution.** The bound rAAV was eluted using 15 ml of elution Buffer B, and the elute was collected in a 50 ml tube [Table 3-3, Step 6]. The rAAV elute was stored at 4°C until the desalting and concentration step was performed.

(4) **Cleaning of Q-Sepharose HP columns.** The columns were allowed to be reused maximum of three times after washing them with 50 ml Buffer A, followed by 50 ml of 20% Ethanol wash. One 5 ml Q-Sepharose HP column was used to purify rAAV isolated from 40-50 plates [15 mm].
Table 3-3 Description of steps of ion exchange column chromatography.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Wash Buffer A 25 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Wash Buffer B 25 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Wash Buffer A 25 ml</td>
</tr>
<tr>
<td>4.</td>
<td>rAAV mixed with Buffer A [1:1]. Eluent reapplied. 6 ml x2</td>
</tr>
<tr>
<td>5.</td>
<td>Wash Buffer A 25 ml</td>
</tr>
<tr>
<td>6.</td>
<td>Elute Buffer B 15 ml</td>
</tr>
</tbody>
</table>

3.6.3.11 Concentration and desalting

The aim of this step is to remove the high salt-containing buffer fraction from the eluent [Buffer B contains 500 mM NaCl] and concentrate the viral stock to a final volume between 200-500 μl using serial centrifugation steps. We used Amicon® Ultra-15 centrifugal filter device [Millipore UFC910008].

Centrifugation steps:

(1) The filter device was washed first with 15 ml of 1x PBS-MK buffer. The buffer was added to the top of the filter and centrifuged at 2000g for 15 minutes. Approximately 500 μl liquid remained above the filter and the rest of the buffer from the 50 ml falcon tube was discarded.

(2) Fifteen ml [15 ml] rAAV containing eluted sample was placed then over the filter and filled up with 15 ml 1x PBS-MK and centrifuged at 2000g for 15-20 minutes. The aim was to obtain 300-500 μl sample above the filter. If the target volume was not achieved the centrifugation step continued.

(3) At this point the ~ 500 μl solution was flushed up and down onto the side of the filter device 5 times in order to re-suspend the viral particles adhered to the filter.

(4) After flushing the filter, 15 ml of 1x PBS-MK buffer was added to the sample and centrifuged at 2000g. The spin time was increased to approximately 10 minutes as the time took successively longer to reduce the sample volume to ~ 300-500 μl.
(5) Step (3) and (4) was repeated three more times.

(6) After the last 1x PBS-MK wash and centrifugation the volume was reduced to 200 μl and the sample pipette up and down on the sides of the filter device to re-suspend the viral particles. Subsequently the samples were aliquoted and stored in the -80°C freezer. Three aliquots of 5 μl were stored in a PCR tube for subsequent determination of rAAV titre.

3.6.3.12 Real time PCR

Polymerase chain reaction [PCR] amplifies DNA exponentially \(2^n\). The number of cycles and the amount of PCR product can be used to calculate the initial quantity of genetical materials. These can be either cDNA or specific sequences within a DNA [in our case the CMV promoter of the IκBα-SR expression cassette]. In real time PCR, the amount of DNA is measured after each cycle by the fluorescent markers [SYBR® Green I] that are incorporated to the PCR product. The increase of change in fluorescent signal is proportional to the number of PCR products generated in the exponential phase of the reaction. The change in fluorescence over the course of the reaction is measured by an instrument which controls the thermal cycles and at the same time is able to scan the process. The machine plots the measured fluorescence against the cycle number that represents the accumulation of product during the entire PCR reaction.

There are three major steps that make up the PCR reaction:

(1) Denaturation: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

(2) Annealing: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. The annealing temperature is about 1-2°C below the melting temperature of the primers used.(3) Extension: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at around 72°C. In our experiment we used HotStart Taq polymerase enzyme with a temperature optimum 60°C. At this
step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding nucleotides that are complementary to the template. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The rate of synthesis occurs generally at a rate of 100 bases per second.

(4) Final hold/soak: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

**Preparation of the rAAV viral sample for real time-PCR:**

1 μl from the viral sample was mixed with 99 μl 1x DNAse buffer [total volume of 100μl]. Subsequently, 350 Units of DNase I [Sigma, D7291; 200 U/μl, therefore 1.7 μl was used] was added to the solution and incubated in the PCR machine for 30 minutes at 37°C followed by 10 minutes at 95°C to denature the DNAse. The samples were centrifuged at 13,000 g for few seconds to ensure that all samples were at the bottom of the PCR tube. 1 μl of Proteinase K [10mg/ml, Qiagen] was added to each sample to digest the viral capsids and the mix was incubated for 60 minutes at 50°C, 20 minutes at 95°C to denature the Proteinase K and 4°C soak. Samples were either used immediately for RT-PCR or stored at -20°C.

**Our real time PCR protocol included:**

(1) Denaturation: 95°C for 2 minutes.

(2) Annealing and extension: 95°C for 15 seconds followed by 58°C for 30 seconds. This sequence [annealing and extension was repeated 40 times].

Analysis of real time-PCR includes the definition of baseline, threshold cycle using the amplification plot [see Chapter 8, Section 8. 3.14.4]. Post amplification melting curve analysis helps to test the presence of artifacts, contamination and to ensure reaction specificity [see Chapter 8, Section 8. 3.14.4]. Serial dilution of known template concentrations can be used to establish a standard curve for determining the original amount of sample [see Chapter 8, Section 8. 3.14].
3.7 DETERMINATION OF IkBα-SR TRANSGENE TRANSDUCTION IN LUNG TISSUE

3.7.1 ASSESSMENT OF TRANSDUCTION EFFICIENCY OF LUNG EPITHELIAL CELLS BY FLOW CYTOMETRY

3.7.1.1 Preparation of single cell suspension from lung tissue

Single cell suspension protocol was adapted and modified based on protocols from the literature[284]. Immediately after exsanguination lung tissue was removed and finely chopped from randomly selected lung areas. Lung tissue was then placed in a 15 ml tube and 3 ml of Collagenase Type I [Sigma-Aldrich; #: C9891] was added [600 units/3 ml of Dulbecco’s Modified Eagle Medium (DMEM)] and digested at 37°C degrees for 2 hours. Tissue preparation was vortexed every 20 minutes. After 2 hours, the thick lung homogenate was filtered to remove debris and the cell suspension was collected in a 50 ml tube. Subsequently 10-20 ml DMEM was used to stop digestion and to wash through the filter and the cell suspension was centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the cell pellet resuspend in 3 ml of red cell lysis [ACK lysis buffer: 150mM NH₄Cl, 10mM KHCO₃ 0.1mM Na₂EDTA] buffer for 2 minutes. After that, 10 ml DMEM was added to dilute red cell lysis buffer and the mix was transferred into a 15 ml tube and centrifuged at 1500 rpm for 10 minutes. Debris free cells resulted in a white cell pellet and subsequently resuspended in 10 ml FACS buffer [1%BSA, 0.1%NaN₃]. The suspension was allowed to stand on ice for 10 minutes. From the top 7 ml cell suspension was filtered again to remove any remaining cell clumps and the sample was stored on ice prior to FACS analysis.

3.7.1.2 Antibody staining of lung epithelial cells from single cell suspension

Two ml of cell suspension [5x10⁶cells/ml] was placed into a 15 ml tube and 40 µl 1:20 concentration of epithelial specific antibody [Anti-pan cytokeratin-11 antibody, #:ab52460; Cambridge, UK] was used to identify lung epithelial cells. The mix was kept on ice and in the dark for 20 minutes and washed with FACS buffer. Staining was performed with ice cold reagents and solutions, since low temperature and presence of NaN₃ [sodium azide] prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity. After 20 minutes the suspension was centrifuged at 1500 rpm for 10 minutes and the cell pellet resuspended in 1 ml FACS buffer.
3.7.1.3 Flow cytometry and analysis of lung epithelial cells

Becton Dickinson FACScan Flow Cytometer [Block Scientific, NY, US] was used to determine specific cell populations from lung samples. Morphological and numerical analysis of single cell suspension was performed by plotting LASER beam side scatter [SSC] values against forward scatter [FSC] values. Subsequently, cells tagged with phycoerythrin-conjugated epithelial specific antibody [Anti-pan cytokeratin-11 antibody, #:ab52460; Cambridge, UK] were identified. This cell subpopulation represented epithelial cells [Type I and II] from the lung homogenate. Fluorescein isothiocyanate [FITC] excitation [488nm] was used to calculate the percentage of lung epithelial cells that were transduced with GFP representing IκBα-SR transduced cells.

3.7.2 REAL TIME-PCR TO DETERMINE IκBα-SR-GFP TRANSGENE INDUCTION AND ENDOGENOUS IκB-α GENE INDUCTION AFTER INJURIOUS VENTILATION

We tested IκBα-SR transgene expression in lung tissue independently from endogenous IκBα expression using real-time PCR. Of note, the transgene IκBα-SR was a human transgene which has similar, but not identical, DNA sequences with endogenous rat IκBα gene. In order to differentiate between the two gene products we used specific human IκBα [HS-IκBα] primers and rat IκBα [RN- IκBα] primers for the PCR reaction.

3.7.2.1 RNA extraction

200mg of lung tissue from each animal’s right lung was used to prepare cDNA. First RNA extraction from each sample was undertaken. Each tissue sample was placed in 15ml tubes and washed with PBS. Homogenization of the tissue was done using a Qiagen TissueRuptor™ [Quiagen, Venlo, Netherlands] with 1ml of TRIzol® reagent [Life Technologies Corporation, #:15596-026, Ireland] added per 100mg of tissue. Once the tissue was completely dissolved, phase separation was undertaken. Chloroform (200µl/ml TRIzol®) was added, vortexed for 15 seconds and left at room temperature for 2-3 minutes. Following centrifugation three phases were visible within each tube. The aqueous top phase was carefully removed to a new tube and isopropanol (500µl/1ml TRIzol®) was added. The samples were left to incubate at room temperature for ten minutes. The samples were then centrifuged at 12,000g for 15
minutes at 4°C. The supernatant was removed and the RNA pellet washed with 80% ethanol per 1ml TRIzol® and vortexed. The samples were again centrifuged at 7500g for 5 minutes at 4°C. The supernatant was removed and the alcohol allowed to air dry for 2-3 minutes. The tubes were transferred to a 70°C heating block and let sit for 2-3 minutes. The pellet was redissolved in 50µl of diethylpyrocarbonate [DEPC] treated water, and heated to 70°C for 5 minutes to fully solubilise. Each sample of RNA was then quantified by Nanodrop spectrometry [Thermo Scientific Nanodrop 2000, Bishop Meadow Road, Loughborough, Leicestershire, UK].

3.7.2.2 cDNA synthesis

The previously prepared RNA was used to produce cDNA and determine IκBα-SR content. cDNA was synthesized from the RNA using the ImProm-II™ reverse transcription system from Promega®, 2800 Woods Hollow Road Madison, WI, USA.

This was carried out as follows:

(1) In tube “A”, 3 µl of the RNA, 1µl of random primer and 1.8 µl water was added. This mixture as incubated at 70°C for 5 minutes and then immediately transferred to ice for 5 min.

(2) In tube “B” the following mixture was prepared. 3.7 µl water, 4 µl Improm-II 5X buffer, 4.8 µl MgCl₂, 1 µl dNTP mix, 0.5 µl recombinant RNasin ribonuclease inhibitor and 1 µl Improm-II RT [reverse transcriptase].

(3) The contents of Tubes “A” and “B” were added to a PCR tube.

(4) The mixture was subjected to the following program in the thermocycler:

- 25°C for 5 minutes,
- 42°C for 1 hr,
- 70°C for 15 minutes.

A negative reaction was also setup. This reaction was the same as above with the substitution of water for RT. The resulting cDNA, was diluted 1:10 was stored at -20°C until use for real time PCR analysis.
3.7.2.3 Real time-PCR

Each cDNA sample was subjected to duplicate analysis. Quantitative PCR was performed for human IκBα-SR and rat IκB-α, normalised against a GAPDH control product. We looked for a comparison of fold induction of human IκBα-SR and rat IκB-α amongst the 3 groups of animals [Vehicle, AAV6-transgene, AAV6-Null], to ensure we had successfully delivered AAV6-IκBα-SR construct to our treatment group.

Creation of the SYBR-Green Real-time PCR reaction mix: There was a total volume of 10µL per reaction. A master-mix was made with the appropriate solutions with 5µL of the master-mix per reaction.

The master mix contained:

(1) Fast SYBR® Green Master Mix [2x concentration; Applied Biosystems]: 5µL

(2) Distilled Water: 3.98µL

(3) Forward primer (100µM): 0.01µL - final concentration is 0.1µM

(4) Reverse primer (100µM): 0.01µL - final concentration is 0.1µM

Primer sequences from MWG-Biotech GmbH:

Quantitative primers: Since originally human IκB-α gene was modified to generate a super-repressor transgene, we used human IκB-α primers to detect the induction of the instilled IκBα-SR transgene. In parallel, endogenous IκB-α gene expression was determined using rat specific IκB-α primers. Primer sequences were from [MWG-Biotech GmbH, Germany].

Human IκBα-SR primers:

Forward sequence: GTC AAG GAG CTG CAG GAG AT

Reverse sequence: CCA TGG TCA GTG CCT TTT CT

Rat IκB-α primers:

Forward sequence: ACG CTG CCC GAG AGT GAG GAT
Reverse sequence: GAG GGA GAA TGG ACC ACT CT

**Rat Lamin A/C primers:**

Forward sequence: AAT GAC CGT CTG GCC CTG TA

Reverse sequence: TCG GCT GAC CAC CTC TTC A

All samples were triplicates. The total volume of master mix was calculated as it corresponded to the number of the samples. The cycle set up in the StepOne Plus Real Time-PCR System [Applied Biosystem] was as follows:

(1) 50 °C for 2 minutes.

(2) 95 °C for 5 minutes.

(3) 95 °C for 15 seconds and 60 °C for 30 seconds.

This cycle was repeated 40 times. Data were analysed by comparing fold inductions calculated from C\textsubscript{T} values.
CHAPTER 4
CHAPTER 4: ESTABLISHMENT OF A PROLONGED SYSTEMIC SEPSIS MODEL AND DETERMINATION OF KEY FACTORS CONTRIBUTING INJURY SEVERITY

4.1 ABSTRACT

Background: Our aim was to develop a prolonged sepsis induced lung injury model and to determine the key factors contributing to injury severity in this model. We used a rodent caecal ligation and puncture [CLP] model for numerous reasons: (1) The model is highly relevant clinically, and the pathophysiological changes triggered by CLP are closely resembles those seen in human systemic sepsis; (2) CLP is a versatile model and it is possible to vary the severity of sepsis; (3) CLP is a simple procedure and the model is easily reproducible.

Methods: Twenty-seven adult, male Sprague-Dawley rats were utilized. We carried out nine experiments using an iterative design whereby the findings in one series informed the design of the subsequent series. In each experiment animals underwent CLP under anaesthesia, were allowed to recover, and were closely monitored and fluid resuscitated. The effect of a number of key factors, including (1) the percentage of the caecum ligated, (2) the size and number of caecal punctures, (3) the amount of fluid administered, and (4) the duration of the model, on animal survival and the severity of lung injury, was determined. Once the model was refined, an initial study of the effect of environmental hypercapnia on injury severity was performed. Animal survival, clinical signs, physiological parameters, BAL neutrophil counts, bacterial growth in blood, BAL and peritoneal fluid were assessed.

Results: Mortality was influenced mainly by the percentage of caecum ligated, and by the size and number of caecal punctures. Fluid administration prolonged survival. All animals developed clinical signs of sepsis. Autopsy revealed peritonitis, necrotized caecum with abscess formation. Animals developed lactate acidosis accompanied with pyrexia or normothermia. Alveolar-arterial O$_2$ difference [DAaO$_2$] was increased and BAL neutrophil counts were elevated after 96h CLP. Blood, BAL and peritoneal fluid bacterial growth confirmed bacteraemia after CLP. We found that ligating 37.5% of the caecum and using two 21g through and through punctures reliably produced
bacteraemia, clinical signs of sepsis, lactate acidosis and lung injury over a 96 hour period. Animals subjected to this injury and subsequently exposed to 8% environmental hypercapnia for 48 or 72h developed HCA, demonstrated less evidence of sepsis and had lower lactate levels than animals subjected to normocapnia.

**Conclusion:** Key factors in determining injury severity in this model include the amount of caecum ligated, the size and number of caecal perforations and the fluid resuscitation protocol. By optimising these parameters, we developed a stable, reproducible 96 hours CLP model.
4.2 INTRODUCTION

Sepsis and sepsis induced acute lung injury continues to be the major cause of death in most critically ill patients [8]. The early, hyper-acute phase of sepsis is well characterized and most of the current therapeutic strategies are aiming to help patients to survive the so called “cytokine storm” and prevent the development of multiple organ failure [285]. However, it has been recognised, that the majority of patients whom survives the initial hyperinflammatory state progress to a prolonged period of immunosuppression associated with secondary infections and high rate of mortality [286].

Hypercapnia - generally “tolerated” to permit reduced intensity mechanical ventilation -, appears to have direct beneficial effects in non-septic lung injury [88, 89, 125, 138]. Recently, new insights into the biological effects of hypercapnia have suggested that the presence of prolonged hypercapnia may be deleterious and worsens lung injury in the context of bacterial pneumonia [133]. Our group has demonstrated in prior studies that HCA in the early phases of CLP induced sepsis and associated lung injury is protective [131]. The biological effect of carbon dioxide on the immune system seems to be inhibitory [135-137], therefore, it is important to clarify the impact of HCA on different phases of sepsis. Specifically, it is important to determine whether HCA might be harmful in the later - functionally immunosuppressed - phase of bacterial sepsis.

Our aim was to develop a prolonged systemic sepsis model in rats expressing both the clinical and pathophysiological features of sepsis. We chose the CLP model, since this model represents best the complex pathophysiological processes in human sepsis [287]. It comprises three main insults: (1) tissue injury due to laparotomy, (2) tissue necrosis secondary to caecal ligation and (3) infection as a result of spillage of bowel content into the peritoneal cavity [248]. In addition, animals undergoing CLP reliably develop bacteraemia and an inflammatory response manifesting clinical signs of sepsis and measurable distant organ dysfunction, including acute lung injury [250]. Most importantly, the severity of the CLP model can be modified and the magnitude of septic insult adjusted to obtain the desired sepsis pattern. However, variability is an
issue with the model – just as in the clinical setting – and it is important to characterize the model variables that contribute to injury severity.

After literature research, we have decided to aim for a 96 hours sepsis model with reasonable mortality rate in order to investigate the effect of sustained HCA. The evolution of inflammation over this time frame seems to mimic both the initial hyperinflammatory phase of sepsis and later the shift toward a hypo-inflammatoty state. However, we had to take into account that more prolonged survival in this model means that the associated organ injury, including the lungs, may be less severe and may results in less impressive changes in lung injury markers.

In these studies, we examined the effect of a number of key factors on animal survival and on the severity of lung injury. These included: (1) length of the caecal ligation [254]; (2) the size and number of caecal punctures [253]; (3) the amount of resuscitation intravenous fluid administered [288]; (4) duration of the septic insult. Once the optimal conditions to produce a reproducible systemic sepsis model were determined, an initial study of the effect of environmental hypercapnia on injury severity was performed.
4.3 METHODS

All experiments were carried out under a licence from the Department of Health and Children, Ireland, and following approval from the Institutional Animal Care Research Ethics Committee of the National University of Ireland, Galway.

Using an iterative design approach, we aimed to develop an in vivo rodent CLP model which features the main characteristics of prolonged abdominal sepsis induced lung injury similar to humans. We carried out nine separate experiments. Observations from each experiment served the basis for further adjustments for the subsequent experimental design. The modified, new experimental design was tested again and the results re-evaluated. Results were then utilized to make subsequent changes for the next experiment. The cycle was repeated until the animal model most closely resembled to the desired model [Figure 4-1].

We used a total of 27 adult, male Sprague-Dawley rats. Since the experimental settings were repeatedly changed during model development, we provide a more detailed description of experimental designs in the Results section [4.4]. Descriptions of measurements are given in the Materials and Methods chapter [Chapter 3, Section 3.5].

4.3.1 ANIMAL SURGERY

Adult male Sprague-Dawley rats were anaesthetised with Isoflurane and ketamine intraperitoneally. The lower half of the abdomen was shaved and disinfected and the caecum was mobilized through an approximately 2cm long, median abdominal incision. The caecum was filled by gently “milking back” colon contents and then ligated with a 3-0 silk ligature distal to the ileo-caecal valve at varying lengths [25, 37.5, 50 %] depending on the experimental plan. The caecum was then subjected to a single or double through and through perforation with a sterile 19 or 21 gauge needle and gently compressed until its contents began to exude, to ensure patency of the perforation sites. The bowel was then repositioned, and the abdominal incision was closed in two layers with 4-0 silk sutures. All animals received normal saline subcutaneously [s.c.] immediately after surgery and allowed to recover from anaesthesia.
4.3.2 TREATMENT PROTOCOL

Once animals had recovered from anaesthesia, they were placed in individually ventilated cages and placed under close observation for differing time period depending on the actual pilot protocol. To test the effect of hypercapnia on animals, 4 animals were exposed to hypercapnia and were housed in an environmental chamber. For these animals ambient oxygen was maintained at 21% and carbon dioxide at 8% using automated controllers. Animal distress was assessed using a composite scoring system [Table 3-1]. An opioid analgesic, Buprenorphine [Temgesic®] 0.01- 0.05 mg/kg was administered postoperatively three times a day to provide adequate pain relief. The amount and the frequency of postoperative fluid management was continuously evaluated and tailored to achieve the desired outcome.

4.3.3 ANAESTHESIA, DISSECTION AND ASSESSMENT OF INJURY

After a pre-determined time period following the CLP procedure, anaesthesia was induced with intraperitoneal ketamine and xylazine. After confirming depth of anaesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein, and further anaesthesia was maintained with an intravenous Saffan infusion. A tracheostomy tube was inserted and secured and intra arterial access was placed in the right external carotid artery. After confirmation of depth of anaesthesia by using paw clamp, Cisatracurium besilate was administered intravenously to produce muscle relaxation. The animals were ventilated with the following parameters: respiratory rate of 90 breaths/min, tidal volume of 6 ml/kg, and PEEP of 2.5 cmH₂O. The animals received an inspired gas mixture of 30% oxygen, and 70% nitrogen. Animals housed in environmental hypercapnia prior to surgery were ventilated with an inspired gas mixture of 5% of carbon dioxide, 30% oxygen and 65% nitrogen. To minimize lung derecruitment, a recruitment manoeuvre consisting of a positive end-expiratory pressure of 10 cmH₂O for 25 breaths was applied after initiation of mechanical ventilation and at the end of the 20 min baseline period. Body temperature was maintained at 36-37.5°C by using a thermostatically controlled blanket system and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures, and temperature were continuously measured throughout the experimental protocol. After 15 min of mechanical
ventilation, an arterial blood gas sample was drawn for blood gas measurement. Subsequently, animals were ventilated for 15 min with FiO$_2$: 1.0 and arterial blood gas sample was taken and BAL total cell counts and neutrophil counts determined.

4.3.4 MEASUREMENTS OF BAL CELL COUNTS AND BACTERIAL COUNTS IN BLOOD, BAL AND PERITONEAL FLUID

Physiological variables were measured as described in the Materials and Methods chapter [Chapter 3, Section 3.5.1]. The tissue sampling, determination of BAL total cell, neutrophil cell count and bacterial colony counts were carried out as described in Materials and Methods chapter [Chapter 3, Section 3.5.2-3.5.4].

4.3.5 DATA ANALYSIS

Data are expressed as mean +/- SD. The purpose of these experiments was to examine the factors that contribute to injury severity in the CLP model. No specific hypotheses were tested, and the analysis is therefore simply descriptive.
4.4 RESULTS

A total of 27 Sprague-Dawley rats were utilized in 9 separate experiments.

4.4.1 EXPERIMENT I. – DETERMINATION OF 48H SURVIVAL FOLLOWING CLP INJURY.

Aim: To achieve 48 hours survival with clinical signs of sepsis and lung injury.

Experimental design: 2 rats underwent 50% cecal ligation and one through and through puncture with a 19 gauge [1x19g] needle. Animals were administered one bolus of 20 ml/kg normal saline s. c. immediately postoperatively [Table 4-1].

Table 4-1 Design of Experiment I.

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of experiment [h]</td>
<td>48</td>
</tr>
<tr>
<td>Percent of cecal ligation [%]</td>
<td>50</td>
</tr>
<tr>
<td>Puncture size and number</td>
<td>1 x 19g</td>
</tr>
<tr>
<td>Postoperative fluid [s.c.]</td>
<td>20 ml/kg</td>
</tr>
</tbody>
</table>

Experimental findings: Both animals died within 24 hours. On observation, animals were appeared septic, had reduced fluid and food intake, piloerection, reduced activity and diarrhoea. Post mortem examination revealed some abdominal faecal soiling, and an inflated and necrotic caecum. The chest cavity and the lung surface contained bacterial inoculations [Table 4-2]

Table 4-2 Findings for Experiment I.

<table>
<thead>
<tr>
<th>Weight [g]</th>
<th>#1 m = 566; #2 m = 520</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival duration [h]</td>
<td>&lt;24</td>
</tr>
<tr>
<td>Autopsy findings</td>
<td>Infected peritoneal fluid, necrotic caecum with abscess formation</td>
</tr>
</tbody>
</table>

Key insights: Early mortality occurred in these animals likely relate to severe sepsis and hypovolaemia.

Proposed changes: For the next experiment, we decided to reduce the percentage of caecum ligated and increased postoperative fluid administration.
4.4.2 EXPERIMENT II. – DETERMINATION OF EFFECT OF REDUCED CAECAL LIGATION AND INCREASED FLUID THERAPY ON ANIMAL SURVIVAL.

**Aim:** To reduce the amount of caecum ligated to achieve 48 hours survival and to carry out physiological measurements to determine the degree of lung injury.

**Experimental design:** 2 rats underwent 25% cecal ligation and 1x19g puncture. Animals were administered one bolus of 20 ml/kg normal saline s. c. immediately after surgery and received another bolus of fluid after 24 hours [Table 4-3].

**Table 4-3** Design of Experiment II.

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of experiment[h]</td>
<td>48</td>
</tr>
<tr>
<td>Percent of caecal ligation [%]</td>
<td>25</td>
</tr>
<tr>
<td>Puncture number and size</td>
<td>1 x 19g</td>
</tr>
<tr>
<td>Immediate postoperative fluid[s.c.]</td>
<td>20 ml/kg</td>
</tr>
<tr>
<td>Regular postoperative fluid [s.c.]</td>
<td>20 ml/kg after 24 h</td>
</tr>
</tbody>
</table>

**Experimental findings:** One of the animals survived the 48 hours experimental period with mild metabolic acidosis and slightly elevated serum lactate level. Arterial blood gases were not abnormal. AaO₂ gradient was slightly elevated compared to values seen in the literature in non septic spontaneously breathing animals [289]. The animal surviving 48h CLP was normothermic, developed bacteraemia, and had increased BAL total cell and neutrophil counts. The second animal died before 36 hours [Table 4-4].

**Table 4-4** Findings for Experiment II.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>#3</th>
<th>#4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>566</td>
<td>520</td>
</tr>
<tr>
<td>Survival [h]</td>
<td><strong>48</strong></td>
<td><strong>36</strong></td>
</tr>
<tr>
<td>Arterial ph</td>
<td>7.34</td>
<td>-</td>
</tr>
<tr>
<td>Arterial pCO₂ [kPa]</td>
<td>5.15</td>
<td>-</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 0.3]</td>
<td><strong>18.7</strong></td>
<td>-</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 1.0]</td>
<td><strong>72.5</strong></td>
<td>-</td>
</tr>
<tr>
<td>Alveolar-arterial O₂ difference [mmHg]</td>
<td><strong>121.4</strong></td>
<td>-</td>
</tr>
<tr>
<td>Serum bicarbonate [mmol/l]</td>
<td>20.8</td>
<td>-</td>
</tr>
<tr>
<td>Base excess [mmol/l]</td>
<td>-4.2</td>
<td>-</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>36.7</td>
<td>-</td>
</tr>
<tr>
<td>BAL total cell count [cell/ml]</td>
<td>32000</td>
<td>-</td>
</tr>
<tr>
<td>BAL neutrophil cell count [cell/ml]</td>
<td>2560</td>
<td>-</td>
</tr>
</tbody>
</table>
Blood bacterial count [CFU x 10^{10}/ml] | 40 | -

**Key insights:** 25% CLP resulted in better survival. However, there was no obvious change in lung physiological injury markers. More fluid replacement may prolong survival after CLP and provide more time to see changes in lung injury markers after CLP.

**Proposed changes:** In the next series we decided to increase the postoperative fluid regime, to determine whether this could increase animal survival.

### 4.4.3 EXPERIMENT III. – EFFECT OF INCREASED POSTOPERATIVE FLUID ADMINISTRATION ON SURVIVAL POST CLP.

**Aim:** (1) to increase fluid resuscitation to achieve 48 hours survival after CLP; (2) to evaluate physiological parameters at 24 and 48 hours.

**Experimental design:** Animals underwent 25% cecal ligation and 1x19g puncture as in the last experiment. All animals had increased fluid regime compared to **Experiment II.** Animals were administered one bolus of 20 ml/kg normal saline s. c. immediately after surgery and received 20 ml/kg 8 hourly postoperatively. One animal was observed for 48 hours and two animals for 24 hours [Table 4-5].

**Table 4-5** Design of Experiment III.

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of experiment [h]</td>
<td>2 animals for 24 or 1 animal for 48</td>
</tr>
<tr>
<td>Percent of cecal ligation [%]</td>
<td>25</td>
</tr>
<tr>
<td>Puncture number and size</td>
<td>1 x 19g</td>
</tr>
<tr>
<td>Immediate postoperative fluid [s.c.]</td>
<td>20 ml/kg</td>
</tr>
<tr>
<td>Regular postoperative fluid [s.c.]</td>
<td>20 ml/kg 8 hourly</td>
</tr>
</tbody>
</table>

**Experimental findings:** The increased fluid regime was associated with 100% survival. There was no major alteration in oxygenation, and animals had similar acid base changes and lactate levels as in the previous series. There was no difference in physiologic indices of injury at 24 versus 48 hours post CLP, and overall the injury was mild. However, as evidenced by blood and BAL bacterial counts, CLP resulted in systemic bacteraemia [Table 4-6].
Table 4-6 Findings for Experiment III.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>#5</th>
<th>#6</th>
<th>#7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>620</td>
<td>602</td>
<td>602</td>
</tr>
<tr>
<td>Experimental duration [h]</td>
<td>24</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.39</td>
<td>7.34</td>
<td>7.38</td>
</tr>
<tr>
<td>Arterial pCO₂ [kPa]</td>
<td>4.73</td>
<td>5.25</td>
<td>4.62</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 0.3]</td>
<td>18.9</td>
<td>19.0</td>
<td>18.6</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 1.0]</td>
<td>76.5</td>
<td>72.5</td>
<td>74.5</td>
</tr>
<tr>
<td>Alveolar-arterial O₂ difference [mmHg]</td>
<td>93.3</td>
<td>121.4</td>
<td>109.3</td>
</tr>
<tr>
<td>Serum bicarbonate [mmol/l]</td>
<td>22.2</td>
<td>20.8</td>
<td>22.4</td>
</tr>
<tr>
<td>Base excess [mmol/l]</td>
<td>-3.1</td>
<td>-4.2</td>
<td>-3.0</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>2.2</td>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>36.7</td>
<td>36.9</td>
<td>36.8</td>
</tr>
<tr>
<td>BAL total cell count [cell/ml]</td>
<td>60 000</td>
<td>46 000</td>
<td>56 000</td>
</tr>
<tr>
<td>BAL neutrophil cell count [cell/ml]</td>
<td>1800</td>
<td>3220</td>
<td>3360</td>
</tr>
<tr>
<td>Blood bacterial count [CFU x 10¹⁰/ml]</td>
<td>16</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>BAL bacterial count [CFU x 10⁶/ml]</td>
<td>0</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Autopsy findings</td>
<td>Infected peritoneal fluid, necrotic caecum with abscess formation.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Key Insights:** Increased postoperative fluid administration was associated with better survival. However, CLP induced lung injury was not marked and metabolic parameters changed moderately, and did not differ between the two time points.

**Proposed changes:** In order to increase the severity of injury, we proposed increasing the extent of caecal ligation for the next experiment.

4.4.4 EXPERIMENT IV. – DETERMINATION OF EFFECT OF LIGATION OF 50% OF THE CAECUM ON SURVIVAL AND SEVERITY OF LUNG INJURY.

**Aim:** To achieve 48 hour survival with more severe lung injury induced by ligation of a larger proportion of the caecum.

**Experimental design:** 2 animals underwent 50% cecal ligation and 1x19g puncture. Animals were administered one bolus of 20 ml/kg normal saline s. c. immediately after surgery and received 20 ml/kg normal saline s. c. 8 hourly postoperatively, i.e. the same fluid regime as in **Experiment III.** [Table 4-7].
### Table 4-7 Design of Experiment IV.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>2</td>
</tr>
<tr>
<td>Duration of experiment [h]</td>
<td>48</td>
</tr>
<tr>
<td>Percent of cecal ligation [%]</td>
<td>50</td>
</tr>
<tr>
<td>Puncture number and size</td>
<td>1 x 19g</td>
</tr>
<tr>
<td>Postoperative fluid [s.c.]</td>
<td>20 ml/kg</td>
</tr>
<tr>
<td>Regular postoperative fluid [s.c.]</td>
<td>20 ml/kg 8 hourly</td>
</tr>
</tbody>
</table>

**Experimental findings:** Increased fluid regime prolonged survival in animals undergoing 50% CLP compared to **Experiment I.**, however, both animals died between 24-36 hours [Table 4-8].

### Table 4-8 Findings for Experiment IV.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>#8 m= 602g; #9 m=560g</td>
</tr>
<tr>
<td>Survival duration (h)</td>
<td>24-36</td>
</tr>
</tbody>
</table>

**Key insights:** 50% caecal ligation causes too severe sepsis and early mortality. In contrast with this, 25% CLP results in stable survival over 48 hours, however, both the resulting sepsis and lung injury is moderate.

**Proposed changes:** In order to increase the septic insult, 37.5 % caecal ligation was applied and fluid administration was increased in the next experiment.

### 4.4.5 EXPERIMENT V. – DETERMINATION OF ANIMAL SURVIVAL TIME FOLLOWING 37.5% CAECAL LIGATION.

**Aim:** To determine animal survival after 37.5% caecal ligation and a further increase in the intravenous fluid resuscitation regimen.

**Experimental design:** 2 animals underwent 37.5% cecal ligation and 1x19g puncture. Animals were administered one bolus of 20 ml/kg normal saline s. c. immediately after surgery and increased fluid regime of 30 ml/kg normal saline s. c. 8 hourly postoperatively. Length of experimental time was unlimited [Table 4-9].

### Table 4-9 Design of Experiment V.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>2</td>
</tr>
<tr>
<td>Duration of experiment [h]</td>
<td>unlimited</td>
</tr>
<tr>
<td>Percent of cecal ligation [%]</td>
<td>37.5</td>
</tr>
<tr>
<td>Puncture number and size</td>
<td>1 x 19g</td>
</tr>
</tbody>
</table>
Postoperative fluid [s.c.] | 20 ml/kg
---|---
Regular postoperative fluid [s.c.] | 30 ml/kg 8 hourly

Experimental findings: 37.5% CLP resulted in stable survival over 120 hours [Table 4-10].

Table 4-10 Findings for Experiment V.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>#10 m = 525; #11 m = 530</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival duration (h)</td>
<td>&gt;120</td>
</tr>
</tbody>
</table>

Key finding: This regimen produces 100% animal survival.

Proposed changes: To increase the chance for stronger injury, we proposed performing 2 separate through and through cecal punctures with a smaller needle [i.e. 2 punctures with a 21g needle]. To counter any increased fluid loss due to the second puncture, we increased fluid administration to 30 ml/kg 6 hourly postoperatively.

4.4.6 EXPERIMENT VI. – TO INVESTIGATE THE EFFECT OF DOUBLE CAECAL PUNCTURE ON SURVIVAL.

Aim: To investigate the effect of double caecal puncture on 48 hour animal survival.

Experimental design: 4 animals underwent 37.5% cecal ligation and two through and through puncture with a 21 gauge [2x21g] needle. Animals were administered one bolus of 30 ml/kg normal saline s. c. immediately after surgery and increased fluid regime of 30 ml/kg 6 hourly postoperatively [Table 4-11].

Table 4-11 Design of Experiment VI.

| Number of animals | 4 |
| Duration of experiment [h] | 48 |
| Percent of cecal ligation [%] | 37.5 |
| Puncture number and size | 2 x 21g |
| Postoperative fluid [s.c.] | 30 ml/kg |
| Regular postoperative fluid [s.c.] | 30 ml/kg 6 hourly |

Experimental findings: All four animals survived the 48h experimental period [Table 4-12].
**Table 4-12** Findings for Experiment VI.

<table>
<thead>
<tr>
<th>Weight [g]</th>
<th>#12 m = 525; #13 m = 530; #14 m = 525; #15 m = 530</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival duration [h]</td>
<td>48</td>
</tr>
</tbody>
</table>

**Key insight:** This experimental regimen produced stable animal survival at 48 hours.

**Proposed changes:** In the next series we proposed to determine the effect of the same experimental design on animal survival and injury severity over a longer time course.

4.4.7 **EXPERIMENT VII. – DETERMINATION OF EFFECT OF THE 37.5% CAECAL LIGATION AND DOUBLE PUNCTURE REGIMEN ON INJURY SEVERITY AND SURVIVAL AT 48-72H.**

**Aim:** To investigate the effect of 37.5% CLP for 48 and 72h duration on animal survival and injury severity.

**Experimental design:** 4 animals underwent 37.5% cecal ligation and 2x21g puncture. Animals were administered one bolus of 30 ml/kg normal saline s. c. immediately after surgery and increased fluid regime of 30 ml/kg normal saline s. c. 6 hourly postoperatively. 2 animals were observed for 48 hours and 3 for 72 hours [Table 4-13].

**Table 4-13** Design for Experiment VII.

| Number of animals | 5 |
| Duration of experiment (h) | 2 animals for 48, 3 animals for 72 |
| Percent of cecal ligation (%) | 37.5 |
| Puncture number and size | 2 x 21g |
| Postoperative fluid (s.c.) | 30 ml/kg |
| Regular postoperative fluid (s.c.) | 30 ml/kg after 6 hourly |

**Experimental findings:** All animals survived 48 hours and 2 out of three animals survived the 72 hours experimental period. Animals exposed to 72 hours CLP had more severe metabolic acidosis, higher serum lactate levels and were pyrexic, representing more severe sepsis. BAL neutrophil counts were similar among the animals. Arterial oxygenation and CO$_2$ levels were not abnormal, A-aO$_2$ gradient was comparable to prior experiments [Table 4-14].
Table 4-14 Findings for Experiment VII.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>#16</th>
<th>#17</th>
<th>#18</th>
<th>#19</th>
<th>#20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>543</td>
<td>490</td>
<td>530</td>
<td>600</td>
<td>549</td>
</tr>
<tr>
<td>Survival [h]</td>
<td>48</td>
<td>48</td>
<td>60-72</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Arterial ph</td>
<td>7.4</td>
<td>7.4</td>
<td>-</td>
<td>7.36</td>
<td>7.46</td>
</tr>
<tr>
<td>Arterial pCO₃ [kPa]</td>
<td>4.39</td>
<td>3.72</td>
<td>-</td>
<td>4.08</td>
<td>3.54</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 0.3]</td>
<td>19.0</td>
<td>19.4</td>
<td>-</td>
<td>18.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 1.0]</td>
<td>73.5</td>
<td>74.0</td>
<td>-</td>
<td>75.9</td>
<td>73.6</td>
</tr>
<tr>
<td>Alveolar-arterial O₂ difference [mmHg]</td>
<td>121.4</td>
<td>-</td>
<td>-</td>
<td>100.5</td>
<td>126.1</td>
</tr>
<tr>
<td>Serum bicarbonate [mmol/l]</td>
<td>22.1</td>
<td>22.1</td>
<td>-</td>
<td>18.5</td>
<td>22.1</td>
</tr>
<tr>
<td>Base excess [mmol/l]</td>
<td>-3.5</td>
<td>-4.0</td>
<td>-</td>
<td>-8.0</td>
<td>-4.2</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>2.3</td>
<td>3.1</td>
<td>-</td>
<td>4.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>37.6</td>
<td>38.0</td>
<td>-</td>
<td>38.4</td>
<td>38.4</td>
</tr>
<tr>
<td>BAL total cell count [cell/ml]</td>
<td>40 000</td>
<td>46 000</td>
<td>-</td>
<td>38000</td>
<td>46 000</td>
</tr>
<tr>
<td>BAL neutrophil cell count [cell/ml]</td>
<td>2800</td>
<td>2300</td>
<td>-</td>
<td>1900</td>
<td>2300</td>
</tr>
<tr>
<td>Blood bacterial count [CFU x 10¹⁰/ml]</td>
<td>84</td>
<td>68</td>
<td>36</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>BAL bacterial count [CFU x 10⁶/ml]</td>
<td>8</td>
<td>24</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Peritoneal fluid bacterial count [CFU x 10¹²/ml]</td>
<td>92</td>
<td>112</td>
<td>92</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

**Key Insight:** Animal survival was stabilized with this experimental design.

**Proposed changes:** Our aim was to extend the observation period to 96h in order to give sufficient time to develop lung injury with more marked physiological changes.

**4.4.8 EXPERIMENT VIII. – DETERMINATION OF EFFECT OF 96 HOURS CLP DURATION ON INJURY SEVERITY.**

**Aim:** To investigate the effect of 96 h CLP in rats.

**Experimental design:** 3 animals underwent 37.5% cecal ligation and 2x21g puncture. Animals were administered one bolus of 30 ml/kg normal saline s. c. immediately after surgery and a fluid regime of 30 ml/kg normal saline s. c. 8 hourly postoperatively [Table 4-15].

Table 4-15 Design of Experiment VIII.

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of experiment [h]</td>
<td>96</td>
</tr>
<tr>
<td>Percent of cecal ligation [%]</td>
<td>37.5</td>
</tr>
<tr>
<td>Puncture number and size</td>
<td>2 x 21g</td>
</tr>
</tbody>
</table>
Experimental findings: All animals survived the 96 h experimental period. Arterial pH was higher than in previous experiments with respiratory and renal compensation. Serum lactate levels were around 3 mmol/l. Arterial pO\(_2\) on FiO\(_2\): 1.0 was slightly lower and DAaO\(_2\) was higher than in previous experiments. BAL neutrophil count was the lowest in this group compared to 48, 72h of CLP [Table 4-16].

Table 4-16 Findings for Experiment VIII.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>#25</th>
<th>#26</th>
<th>#27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>410</td>
<td>331</td>
<td>354</td>
</tr>
<tr>
<td>Survival [h]</td>
<td>96</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Arterial ph</td>
<td>7.42</td>
<td>7.48</td>
<td>7.47</td>
</tr>
<tr>
<td>Arterial pCO(_2) [kPa]</td>
<td>5.53</td>
<td>4.8</td>
<td>4.45</td>
</tr>
<tr>
<td>Arterial pO(_2) [FiO(_2): 0.3]</td>
<td>19.3</td>
<td>18.9</td>
<td>18.8</td>
</tr>
<tr>
<td>Arterial pO(_2) [FiO(_2): 1.0]</td>
<td>71.9</td>
<td>69.7</td>
<td>71.1</td>
</tr>
<tr>
<td>Alveolar arterial O(_2) difference [mmHg]</td>
<td>122.8</td>
<td>143</td>
<td>138.3</td>
</tr>
<tr>
<td>Serum bicarbonate [mmol/l]</td>
<td>26.4</td>
<td>27.9</td>
<td>25.8</td>
</tr>
<tr>
<td>Base excess [mmol/l]</td>
<td>2.3</td>
<td>3.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>3.0</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>37.2</td>
<td>36.7</td>
<td>37</td>
</tr>
<tr>
<td>BAL total cell count [cell/ml]</td>
<td>54 000</td>
<td>45 000</td>
<td>39 000</td>
</tr>
<tr>
<td>BAL neutrophil cell count [cell/ml]</td>
<td>1620</td>
<td>2025</td>
<td>1560</td>
</tr>
</tbody>
</table>

4.4.9 EXPERIMENT IX. – DETERMINATION OF EFFECT OF HYPERCAPNIA ON INJURY SEVERITY.

Aim: To investigate the effect of hypercapnia in animals undergoing 37.5% CLP.

Experimental design: 4 animals underwent 37.5% cecal ligation and 2x21g puncture. After surgery animals were exposed to 8% environmental hypercapnia for 48 or 72 hours. All animals received one bolus of 30 ml/kg normal saline s. c. immediately after surgery and 30 ml/kg normal saline s. c. 6 hourly postoperatively. 2 animals were observed for 48 hours and 2 for 72 hours [Table 4-17].

Table 4-17 Design of Experiment IX.
**Table 4-18** Findings for Experiment IX.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>#21</th>
<th>#22</th>
<th>#23</th>
<th>#24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>496</td>
<td>592</td>
<td>610</td>
<td>595</td>
</tr>
<tr>
<td>Survival [h]</td>
<td>48</td>
<td>48</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.36</td>
<td>7.30</td>
<td>7.28</td>
<td>7.26</td>
</tr>
<tr>
<td>Arterial pCO₂ [kPa]</td>
<td>7.50</td>
<td>7.36</td>
<td>8.32</td>
<td>7.61</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 0.3]</td>
<td>22.3</td>
<td>19.9</td>
<td>19.8</td>
<td>21.1</td>
</tr>
<tr>
<td>Arterial pO₂ [FiO₂: 1.0]</td>
<td>80.1</td>
<td>74.9</td>
<td>77.5</td>
<td>78.0</td>
</tr>
<tr>
<td>Alveolar-arterial O₂ difference [mmHg]</td>
<td>69.2</td>
<td>103.4</td>
<td>76.8</td>
<td>-</td>
</tr>
<tr>
<td>Serum bicarbonate [mmol/l]</td>
<td>24.9</td>
<td>24.0</td>
<td>25.1</td>
<td>22.4</td>
</tr>
<tr>
<td>Base excess [mmol/l]</td>
<td>1.9</td>
<td>0.8</td>
<td>2.7</td>
<td>-0.9</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>2.1</td>
<td>3.5</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>36.5</td>
<td>37.3</td>
<td>37.6</td>
<td>37.4</td>
</tr>
</tbody>
</table>

**4.4.10 COMPARISON OF NORMOCAPNIA VERSUS HCA IN ANIMALS FOLLOWING CLP FOR 48 AND 72 HOURS**

**Aim:** To describe the effect of prolonged hypercapnia on survival and physiological changes in animals undergoing CLP for 48 and 72 hours.

**Description of analysis:** Data were retrospectively compared from Experiment VII [Normocapnia] and Experiment IX [HCA]. In Experiment VII, 2 animals were observed for 48 and 3 animals for 72 hours. In Experiment IX 2-2 animals were used in the 48 and 72h groups. Mean ± SD is presented for each variable.
Survival: All animals survived in the HCA group [Table 4-18], while in the Normocapnia group, 2 out of three animals survived the 72h CLP induced sepsis [Table 4-14].

Acid base: Animals exposed to environmental hypercapnia had higher arterial pCO₂ levels [Figure 4-2] and lower pH compared to Normocapnic animals at each time point [Figure 4-3]. Of note, animals surviving 72h CLP under hypercapnia had the lowest pH. Arterial HCO₃ levels were much higher in the HCA than in the Normocapnia group [Figure 4-4]. Base excess values were positive in the HCA and negative in the Normocapnia group [Figure 4-5]. Serum lactate levels were comparable at 48h between the HCA and Normocapnic animals, however, at 72 hours, animals receiving prolonged hypercapnia had decreased lactate values compared to normocapnia [Figure 4-6].

Oxygenation: Arterial oxygen tension was higher in the HCA group than in the normocapnia group at both time points [Figure 4-7]. Hypercapnic animals had also higher oxygen partial pressure when FiO₂ of 1.0 was administered [Table 4-18].

Body temperature: Normocapnic animals were pyrexial both at 48 and 72h suggesting ongoing acute inflammation. In contrast with this hypercapnic animals had normal body temperature [Figure 4-8].
4.5 DISCUSSION

Caecal ligation and puncture [CLP] model for sepsis is one of the models that most closely replicates the nature and the clinical course of intra-abdominal sepsis in humans [249, 250, 287]. The model can be described by the following characteristics: (1) polymicrobial sepsis caused by microorganisms of faecal origin; (2) focal infection in origin; (3) positive blood culture; (4) presence of metabolic and organ dysfunctions similar to human sepsis. In addition, fluid [249, 288] and/or antibiotic [256] administration or surgical intervention [255] after CLP, as they occur in clinical practice, further support the usefulness of this model in sepsis research. However, the most important feature that makes CLP model attractive is that, it is possible to tailor the magnitude of the inflammation to produce a full spectrum of severity [290].

We have developed a sepsis model using CLP in adult, male Sprague-Dawley rats in order to investigate the effect of hypercapnia in the context of prolonged sepsis induced lung injury. Although, antibiotic treatment delays mortality in CLP induced sepsis in rodents [372, 373] and would represent a clinically more relevant sepsis model, we have decided not to use any therapeutic measure in our CLP model. The rational for this was based on the following observations: (1) antibiotic administration does not prevent the development of sepsis induced ALI, however, it delays the manifestation of lung inflammation [374]; (2) antibiotic treatment also interferes with the immune response therefore it may mask the effect of hypercapnia on inflammation particularly in a CLP model with prolonged, mid-grade [moderate] sepsis; (3) our primary hypothesis and research question was to dissect the effect of hypercapnia in an animal model which was not modified by additional therapeutic interventions [either antibiotic administration or surgical removal of the ligated caecum] in order to reduce confounding variables; (4) this approach is also accepted in the literature when the scientific enquiry is directed to prove the effect of a single intervention [proof of concept studies]; (5) survival/outcome after CLP is very variable in rodents even without antibiotics [375], therefore a simpler model is more suitable to investigate the effects of hypercapnia in sepsis and sepsis induced ALI.
Our primary consideration to determine the endpoints of our CLP model were: (1) to develop a 96 hours prolonged sepsis model with reasonable mortality; (2) the strength of the septic insult had to be sufficient to result in measurable sepsis induced ALI after 96 hours. The main challenge in the development of CLP model was to find the right balance between injury strength, survival, and severity of ALI since these endpoints inherently work against each other.

4.5.1 EFFECT OF EXTENT OF CAECAL LIGATION ON SEPSIS SEVERITY AND SURVIVAL

Caecal ligation alone in rodents does not produce peritonitis [248]. In this situation the caecum becomes necrotic or forms an abscess and the inflammation remains localized. In general, this is well tolerated by rodents and quite frequently caecal ligation results in total absorption of the necrotic mass. This has been shown in earlier studies whereby lack of systemic bacteraemia in a caecal ligation model resulted in a very different inflammatory pattern than humans [248]. However, when caecal puncture was added, the resulting faecal soiling caused high mortality, and was accompanied by sepsis like symptoms. [248].

Singleton et al have shown first that one of the major determinants of mortality in CLP induced sepsis is the length of caecum ligated [254]. This may be explained, by the fact, that larger caecum ligation means larger amount of bowel content [i.e. more bacterial load] which can spill out through the puncture site to the peritoneal cavity causing peritonitis and bacteraemia. Singleton et al examined the effect of various percentages of caecum ligation in rats ranging from 5 to 35% [5, 10, 20, 25, 30 and 35%] over 96 hours. The group showed that 5-10% ligation had very little impact on mortality, 20 % of ligation resulted in 40% mortality, and above 25% ligation mortality accelerated and reached around 90-100% on day 4. Greater percentages of caecal ligation resulted in increased mortality and were clearly associated with higher TNF-α and IL-6 levels. Others proposed the application of standardized CLP protocols to control for sepsis severity [290]. Part of this protocol described 3 ranges of CLP ligation which could represent 3 different degree of injury. Low grade sepsis can be evoked by 10% CLP featured with a 90-100% survival at 96 hours, while mid grade sepsis caused by 50% ligation results in 50% survival between day 1-2, however mortality may
stabilize at this level for several days. In contrast with this, animals undergoing caecal ligation of 75% or more, develop rapid sepsis and have around 80% mortality at 96 hours [high grade sepsis].

Our findings were comparable to that described in the literature. We found that 50% caecal ligation resulted in severe sepsis and animals died between 24-36 hours. In contrast with this, 25% ligation resulted in nearly 100% survival at 48 hour with moderate metabolic acidosis and moderately elevated lactate levels. Most importantly, animals had no significant changes in arterial oxygenation, although they did develop alveolar neutrophil infiltration, suggesting some degree of lung injury. To increase the septic insult, but at the same time trying to avoid early mortality, we ligated the caecum at 37.5%. This experimental setting resulted in 100% survival at 96 hours, animals had elevated lactate levels [highest among the experiments], compensated metabolic acidosis and marginally lower arterial oxygenation and elevated BAL neutrophil content. Static compliance was similar to values which we have seen in our laboratory in animals previously with no lung injury.

4.5.2 EFFECT OF CAECAL PUNCTURE SIZE AND NUMBER ON SEPSIS SEVERITY AND SURVIVAL

Wichterman et al suggested first that changing caecal puncture size and number may modify the evolution of sepsis in a rodent CLP model [248]. The investigators demonstrated that increasing needle size from 22g to 18g increased mortality from 23% to 30% in the first day after CLP however, after 72 hours mortality rates were similar, reaching 70-90%. Using 2 punctures instead of one in the 18g needle group, mortality was increased to 75% in the first 24 h and remained high [95%] over time. These observations suggest that puncture size/number influences mainly the early, rather than the late stage of sepsis. These were confirmed by Oterón-Anton et al using puncture size ranging from 0.5 cm blade incision to 22g [0.5 cm, 13, 16, 18, 22g]. Mortality decreased from ~ 90 to 22% according to puncture size in the first 48 hours and then remained stable for the following 5 days. The authors also demonstrated that early endotoxin and TNF-α levels were higher when puncture size was larger after CLP [253].
In attempt to prolong survival and at the same time produce a more severe lung injury we reduced the size of the needle from 19g to 21g and increased the puncture number to 2 in the 37.5% series. These resulted in 100% survival at 48 hours, however, at 72 hours 2 animals from 3 survived the CLP protocol. In parallel, the amount of postoperative fluid was increased to enhance survival therefore it is unclear whether improved survival was attributed to manipulation of puncture size and number per se, or fluid administration. Taken together we can conclude that needle size influences mortality mainly in the early phase of CLP.

4.5.3 EFFECT OF FLUID ADMINISTRATION ON SURVIVAL

CLP induced sepsis has a very distinct mortality pattern compared to other sepsis models [257, 261, 287]. It seems that most of the animal death occurs in the early phase of sepsis somewhere between 12 to 48 hours. If animals survive this early phase, mortality becomes stable and in certain cases animals stay alive for a prolonged period of time or even completely recover. This has been demonstrated in various animal species, including mice [251], rats [254] and pigs [291]. Studies investigating the effect of fluid resuscitation in CLP induced sepsis models have shown that the main impact on mortality was actually seen in this early phase [251, 291]. Zanotti-Cavazzoni et al demonstrated that increasing fluid regime was associated with improved cardiovascular performance in the first 72h after CLP [288]. Most importantly, 48h survival was 75% in the high fluid regime group, 58% in the intermediate fluid resuscitation group and 35% in the low fluid regime group. Of note, stroke volume [SV] and cardiac output [CO] was similar in all three groups at 72 hours and this was the time point when mortality values plateaued in each group. This may indicate that once animals survive the initial shock phase of sepsis, other mechanisms assume greater importance in determining late mortality.

With relevance to our CLP model, we sequentially increased both the amount and number of fluid boluses administered in order to achieve a viable 96h model. Immediate postoperative fluid administration is widely accepted in the literature to help to overcome the initial low cardiac output state in the first 6 hours after CLP and to replace surgical fluid losses [255, 256, 288, 292]. Based on this, we administered 20
ml/kg normal saline [NS] bolus s. c. after surgery in Experiment I-V. We noticed that lack of regular postoperative fluid regime was associated with higher mortality [Experiment I-II] therefore we added 20 ml/kg NS s. c. 8 hourly after 50 and 25% CLP [Experiment III-IV]. This increased survival, and regular postoperative fluid administration was integrated to the protocol. Since our aim was to increase sepsis severity balanced with acceptable survival, once ligation was increased to 37.5%, the regular postoperative fluid was also increased to 30 ml/kg 6 hourly [Experiment V-VIII]. We further modified the immediate postoperative fluid administration [to 30 ml/kg NS s.c.] to compensate for increased sepsis due to increased puncture size [Experiments VI-IX]. In our final model, we used 30 ml/kg NS postoperatively, and 30 ml/kg NS 8 hourly s.c. This was associated with 100% survival at 96 hours in animals undergoing 37.5% caecal ligation with 19g double puncture [Experiment VIII].

In summary, we conclude that the following points need to be considered when designing a fluid regime to achieve a prolonged CLP model: (1) immediate postoperative fluid administration would compensate for surgical fluid loss and initial septic cardiovascular instability; (2) regular postoperative fluid would improve survival and reduce early mortality up to 48-72 hours; (3) the amount of fluid needs to be tailored depending on sepsis severity [i.e. percentage of caecal ligation and puncture size/number]; (4) our CLP model is considered low-mid grade sepsis model according to Rittirsch et al publication [290], therefore, an intermediate fluid regime suggested by Zanotti-Cavazzoni et al [~30-35 ml/kg 6-8 hourly] seemed to be sufficient to realize a 96h CLP model; (5) regular s.c. normal saline administration of such volumes were not stressful for the animals and were well tolerated.

4.5.4 CLINICAL COURSE OF SEPSIS IN A RODENT MODEL OF CLP

Although the severity of the symptoms can vary, generally the following clinical picture emerges in rodents after CLP: lethargy, reduced physical activity and cessation of grooming behaviour, piloerection, glazed eyes with crusting exudates, diarrhoea, reduced fluid and food intake, weight loss, pyrexia or more frequently hypothermia [249, 250]. The hyperacute phase of sepsis presents usually 6 hours after CLP and it is characterized by bacteraemia, endotoxaemia, elevated TNF-α, IL-6 levels and pyrexia
and transient low cardiac output state [288]. This period is followed by hypermetabolism and hyperdynamic circulation up to 16 hours. Without treatment, the majority of animals slip into a hypodynamic, hypometabolic phase accompanied with high blood lactate levels, low white cell counts and die early. In contrast, animals which survive the acute “cytokine storm” [hyperinflammatory response] may succumb to a more protracted immunodepressive phase, characterized by secondary or opportunistic infection [293, 294]. Muenzer et al demonstrated that animals surviving the initial hyperinflammatory response after CLP develop a temporal immunosuppressive period which lasts up to day 7 post CLP [294]. This is evidenced by decreased or total depletion of innate immune cells in the spleen and reduced splenocytes INF-γ production. In surviving animals these changes partially recovers by day 7. Muenzer et al also demonstrated that a second hit injury induced by P. aeruginosa pneumonia on day 4 post CLP, but not on day 7, resulted in worst survival, reduced host response, increased bacterial growth and caused more severe histological injury in the lung [294].

In our 96h CLP model, all animals expressed similar clinical symptoms and dynamics of sepsis as it has been described in the literature. As a result of regular fluid administration tailored to caecal ligation and puncture [Table 4-16], all animals survived the 96h experimental period. Biochemical changes demonstrated compensated metabolic acidosis [renal and respiratory] with high lactate levels [Table 4-16]. Arterial paO₂ on FiO₂: 0.3 seemed to be normal [18.8-19.3 kPa], however, paO₂ levels on FiO₂: 1.0 were slightly less compared to previous experiments [69.7-71.9 kPa]. Alveolar-arterial O₂ difference was increased compared to normal uninjured values seen in the literature [289]. Although we did not measure bacterial growth in Experiment VIII., we did reliably demonstrate increased bacterial growth in blood, BAL and peritoneal fluid samples in Experiment II., III., VII. In addition, all animals underwent autopsy and had, although in varying degree, infected peritoneal fluid with moderate amount of faecal content and abscess formation.

In summary, our 96h CLP model resulted in mild sepsis and sepsis induced lung injury in rats. Generation of a more severe sepsis model, however, would result in early mortality and prevent us to investigate the effect of hypercapnia in prolonged sepsis.
Given the importance of the possible risks of “permissive hypercapnia” in the context of late sepsis when immunosuppression may dominate, our 96h timeframe seems to be clinically relevant to investigate the safety of prolonged hypercapnia.

4.5.5 CLP SEPSIS INDUCED LUNG INJURY

Most of the studies describing lung injury severity after CLP have focused in two time periods: (1) 6-12h [210, 295] and (2) 18-48h [250, 296-299]. Abnormal lung permeability after CLP appears as early as 6h evidenced by elevated BAL total protein concentration [297, 300], increased albumin extravasation, and increased wet to dry lung ratio [299]. Lung histopathological changes include early signs of oedema, diffuse neutrophil and monocyte/macrophage infiltration. BAL neutrophil, monocyte/macrophage or lymphocyte counts are also elevated after CLP compared to sham animals [296, 298]. NF-κB activity in lung tissue and NF-κB dependent cytokines, such as TNF-α and CINC, are also elevated in early sepsis induced ALI [295]. It is important to note, that lung injury evoked by systemic sepsis is generally less severe than in animal models representing direct pulmonary insults, such as inhaled endotoxin [126] or live bacteria induced lung injury [42]. In addition, very little data are available about the nature of lung injury in animals surviving the hyperacute phase of sepsis.

Although lung injury was mild in our 96h CLP model, animals had increased alveolar-arterial O₂ difference [AaDO₂], decreased paO₂ levels on FiO₂ of 1.0 and increased BAL neutrophil counts [Table 4-16]. We have also demonstrated that bacteria were present in BAL after CLP [Experiment II, VII]. We did not perform lung histological analysis, since these were on-going pilot experiments with the aim to demonstrate pathophysiological changes after CLP. It is well described in the literature that the magnitude of CLP induced ALI is variable and depends on the strength of the inciting injury. As it has been mentioned earlier, most of the experimental data describing sepsis induced ALI uses short term models with significant septic insult. This is not surprising, since it is well known that researchers use massive injuries which may be non-representative to that seen in human conditions in order to answer to specific scientific questions [i.e. proof of concept studies]. The trade off between acceptable
survival and sepsis severity is that the evoked sepsis and ALI may be mild, and consequently, detecting changes in injury markers in response to hypercapnia might be difficult. More appropriate clinical scenario would be to evoke a strong septic insult and than keeping animals alive in an “intensive care unit” for 4 days, however, this would be very difficult to achieve in a rodent model.

4.5.6 EFFECT OF HCA ON PHYSIOLOGICAL PARAMETERS IN CLP INDUCED SEPSIS

We have assessed in a pilot experiment how septic animals react to sustained hypercapnia [Experiment VIII]. After CLP, animals were placed in a chamber and exposed to 8% environmental hypercapnia for either 48 or 72 hours. Survival, physiological parameters, and serum lactate were measured. All animals developed hypercapnia, however, hypercapnia and acidosis was more severe at 72 hours than at 48 hours [Table 4-18]. Serum lactate levels were marginally lower in the 72h than the 48h group. All animals survived the experimental period, and expressed fewer septic clinical signs, behaved livelier than normocapnic animals in prior experiments. Arterial oxygenation was slightly higher in the 72h group compared to the values from the 48h group.

We also compared retrospectively Experiment VII with Experiment VIII to analyse the difference between septic animals exposed to normocapnia [Experiment VII] or hypercapnia [Experiment VIII]. Hypercapnic acidosis suggested a survival benefit in septic animals [Table 4-18] and this was associated with lower lactate levels among animals in the 72h group [Figure 4-6]. Arterial oxygenation was also minimally higher in the HCA group at both time points [Figure 4-7]. Animals were pyrexial under normocapnic condition, while remained normothermic under HCA [Figure 4-8].

Our group has previously reported that HCA attenuated early septic shock and sepsis induced ALI in a rodent CLP model [131, 187]. In these experiments 50% caecal ligation and 1x18g caecal puncture was used to induce severe sepsis. In these short experiments [3 and 6 hours], survival, mean arterial pressure, oxygenation and lung injury markers were significantly better when animals were exposed to hypercapnia. In our moderate sepsis model animals reliably developed HCA following exposure to environmental hypercapnia over 72 hours. Although differences in physiological
markers were very modest between hypercapnic and normocapnic animals, our data suggested that HCA may influence outcome after prolonged sepsis induced by CLP.

4.5.7 CONCLUSIONS

1. Following 37.5% caecal ligation and 2x21g puncture, rats reliably develop clinical signs of sepsis, bacteraemia, and biochemical alterations equivalent with lactate acidosis. Increased BAL neutrophil counts suggest ongoing lung injury.

2. A fluid administration regimen that comprises a fluid bolus immediately postoperatively [30 ml/kg normal saline s.c] followed by 8 hourly boluses [30 ml/kg normal saline s. c.] prolongs animal survival.

3. The present CLP model results in good survival rates, with a measurable, if moderate, degree of lung injury.

4. Animals post CLP exposed to 8% environmental hypercapnia for 72 hours develop hypercapnic acidosis and renal buffering. The extent of the hypercapnic acidosis developed is clinically relevant.

In summary, the main advantages of our model are: (1) CLP is widely accepted and the most representative model for systemic human sepsis; (2) The 96 hours timeframe allows us to investigate the effect of prolonged hypercapnia on the injury phase when the function of host immunity might be an important determinant of outcome; (3) this is a reproducible model. The main limitations of this model that CLP sepsis and sepsis induced ALI is relatively mild, therefore to detect changes in injury markers might be difficult.
4.6 TABLES AND FIGURES

Figure 4-1 Schematic overview of iterative experimental design process. The initial experimental model is tested and evaluated. Conclusion from the obtained results leads to the modification of the original animal model. The new model is tested again. The cycle is repeated until the animal model is most closely resembles to the desired model.
Figure 4-2 Final arterial pCO₂ values after 48h and 72h CLP. Animals housed in 8% environmental hypercapnia had higher pCO₂ levels than normocapnic animals.

Figure 4-3 Final arterial pH after 48h and 72h CLP. Animals housed in 8% environmental hypercapnia had lower pH than normocapnic animals. pH was the lowest in animals exposed to 72 h hypercapnia.
Figure 4-4 Final arterial HCO$_3$ values after 48h and 72h CLP. Animals housed in 8% environmental hypercapnia had higher HCO$_3$ values than normocapnic animals indicating the presence of renal buffering.

Figure 4-5 Final arterial BE values after 48h and 72h CLP. Animals housed in 8% environmental hypercapnia had positive, while normocapnic animals had negative BE values.
Figure 4-6 Final arterial lactate values after 48h and 72h CLP. At 72h lactate levels decreased in animals housed in 8% environmental hypercapnia while normocapnic animals had increased lactate levels.

Figure 4-7 Arterial oxygenation was marginally higher under hypercapnic condition both at 48h and 72h compared to normocapnia.
Body temperature was high in animals after 48h and 72h of normocapnia. Hypercapnia was associated with lower temperature in animals undergoing CLP both at 48 and 72h.

**Figure 4-8** Body temperature was high in animals after 48h and 72h of normocapnia. Hypercapnia was associated with lower temperature in animals undergoing CLP both at 48 and 72h.
CHAPTER 5: DETERMINATION OF EFFECTS OF HYPERCAPNIC ACIDOSIS ON THE SEVERITY OF PROLONGED SYSTEMIC SEPSIS AND THE ROLE OF THE NF-κB PATHWAY IN MEDIATING THESE EFFECTS

5.1 ABSTRACT

Background: Prolonged exposure to hypercapnia may suppress immune function and inhibit host bacterial killing. It has been recently reported that sustained HCA worsened bacterial pneumonia and increased bacterial load in an *E.Coli* induced ALI model. HCA also inhibits the antimicrobial activity of immune cells both *in vitro* and *in vivo*. Furthermore, the effect of HCA seems to be mediated by inhibiting the NF-κB pathway. Overall, these data indicate that prolonged exposure to HCA might be deleterious and could potentially suppress the immune response to invading pathogens. Therefore, we wished to investigate the effect of prolonged environmental hypercapnia in CLP sepsis and sepsis induced lung injury.

Methods: Adult male Sprague-Dawley rats were utilised. Animals were anaesthetised and underwent caecal ligation and puncture. After recovery from anaesthesia, the animals were randomised to receive hypercapnia [FiCO$_2$:0.08, FiO$_2$:0.21, n=20] or normocapnia [FiO$_2$:0.21, n=20] in an environmental chamber and survival rate determined. After 96 hrs, 8 animals in each group underwent surgery and lung physiological parameters, BAL total protein and specific cytokine, lung histological injury markers and the activity of the NF-κB pathway were assessed.

Results: 96 hour survival was similar between the two groups. Prolonged hypercapnia did not worsen sepsis and did not increased bacterial load in BAL, blood and peritoneal fluid. There was no difference in oxygenation, lung static compliance, permeability, and BAL inflammatory markers when animals were exposed to hypercapnia or normocapnia. HCA did decreased histological injury compared to normocapnia. HCA also inhibited the NF-κB pathway evidenced by decreased p65 DNA binding activity in lung tissue homogenates.

Conclusion: Sustained hypercapnia did not worsen sepsis and did not increase bacterial load in a CLP model. In addition, hypercapnia resulted in moderate protection
in sepsis induced lung injury as evidenced by reduced histological injury indices and NF-κB activity.
5.2 INTRODUCTION

Sepsis is a major cause of morbidity and mortality in critically ill patients, accounting for more than 210,000 deaths in the U.S. alone [31]. It has been increasingly recognised over the last decade that the immune reaction in response to sepsis is complex, and undergoes phases which shift from the initial acute, hyperinflammatory [systemic inflammatory response syndrome: SIRS] toward a more prolonged immunosuppressive state [compensatory anti-inflammatory response syndrome CARS] [286, 293]. Furthermore, the balance between these two opposing pathophysiological processes are greatly influenced by patient specific factors, such as age, gender, nutritional status, presence of co-morbidities and the genetic make-up of the individual. Earlier scientific efforts and current therapies in sepsis have been focused in the management of the initial phase of sepsis resulting in a large patient population whom survive. However, some of these patients may enter into a prolonged hypo-inflammatory state characterised by severe immunoparalysis. Recent research to this area indicates that patients in whom immune function cannot recover are unable to clear pathogens and have an increased risk to nosocomial infections. Two important consequences arise from these observations: (1) most deaths in sepsis are related to this immunosuppressed phase [301]; (2) therapeutic strategies or clinical states which promote or result in immunosuppression may be devastating in this patient population.

Protective lung ventilation and permissive hypercapnia are integral parts of the management of septic patients. The importance of the effect of HCA in this patient population is highlighted by large number of in vivo and ex vivo studies suggesting that CO₂ is a potent immunomodulatory agent and may reduce ALI induced by various insults [42, 88, 89, 126, 127, 129, 131]. Initial preclinical studies using HCA in the acute pro-inflammatory phase of sepsis [both pulmonary and systemic sepsis] was found to be protective. This protective effect of HCA seems to be linked to its anti-inflammatory properties via inhibition of cytokine signalling, phagocyte function, and adaptive immune responses [42, 72, 136, 302]. In contrast, in the context of prolonged sepsis, when immunosuppression may dominate and determine outcome, the acceptance of sustained hypercapnia might be deleterious by inhibiting the overall immune response
to invading pathogens [141]. The potential of HCA to exert deleterious effects in the context of live bacterial infection was demonstrated first in a prolonged bacterial pneumonia model [133]. In this experiment bacterial load was associated with impaired neutrophil phagocytic activity under hypercapnic condition, which is critical to bacterial clearance. This issue is of crucial importance because nosocomial infections are commonly present in critically ill patients whom might be exposed to sustained hypercapnia as a result of COPD, or prolonged ventilation with lung protective ventilation strategy.

The NF-κB pathway is one of the key regulators of the complex inflammatory response in sepsis and ALI/ARDS. This has been supported by both preclinical and clinical studies showing: (1) NF-κB pathway is activated in multiple cells [immune, endothelial, and epithelial] in response to various stimuli including endotoxin, live bacteria, oxidative stress, mechanical stretch, ischaemia-reperfusion, hyperoxia etc., and this is associated with increased NF-κB dependent cytokine release [96, 190, 209-213, 303] (2) macrophages and neutrophils from patients with sepsis and sepsis induced ALI have increased NF-κB activity [204, 206]; (3) selective induction of NF-κB pathway via gene transfer results in acute lung injury [209]; (4) inhibition of NF-κB pathway reduces inflammation and cytokine release [96, 190, 210-213] and attenuates the clearance of pathogen organisms [214] and may reduce tissue repair [134, 191]. The potential of HCA to exert both beneficial and deleterious effect may be related to its inhibitory effect on the NF-κB pathway. HCA has been shown to inhibit LPS induced injury in vitro in lung endothelial cells by preventing IκB-α degradation [190], however, NF-κB pathway inhibition by HCA also resulted in delayed epithelial wound repair [134]. In addition, CO₂ mediated NF-κB inhibition seems to attenuate host response in Drosophila and to reduce the phagocytic activity of human alveolar macrophages in vitro [135-137].

Our group and others have previously demonstrated that HCA attenuates the hemodynamic effects of early systemic sepsis and sepsis induced ALI [131, 132]. Based on the aforementioned reasons, we wished to investigate the effect of hypercapnia in animals exposed to environmental hypercapnia for 96 hours following CLP induced
sepsis. We also wished to determine how hypercapnia influences NF-κB pathway activity in sepsis induced ALI. We hypothesised that prolonged HCA would worsen non pulmonary sepsis and sepsis induced lung injury.
5.3 METHODS
A total of 40 specific-pathogen-free adult, male Sprague-Dawley rats were used. All experiments were carried out under a licence from the Department of Health and Children, Ireland, and following approval from the Institutional Animal Care Research Ethics Committee of the National University of Ireland, Galway.

5.3.1 ANIMAL SURGERY
Adult male Sprague-Dawley rats were anaesthetised with isoflurane gas and intraperitoneal ketamine injection. The lower half of the abdomen was shaved and disinfected and the caecum was mobilized through an approximately 2-cm-long, median abdominal incision. The caecum was filled by gently “milking back” colon contents and then ligated with a 3.0 silk ligature distal to the ileo-caecal valve at 37.5%. The caecum was then subjected to a double through and through perforation with a sterile 21-gauge needle and gently compressed until its contents began to exude, to ensure patency of the perforation sites. The bowel was then repositioned, and the abdominal incision was closed in two layers with 4.0 silk sutures. All animals received 30 ml/kg normal saline s.c. immediately after surgery and allowed to recover from anaesthesia.

5.3.2 TREATMENT PROTOCOL
After recovery from anaesthesia, animals were randomised to be housed under conditions of environmental hypercapnia or normocapnia for 96 hours. Animals randomised to hypercapnia were housed in an environmental chamber in which ambient oxygen was maintained at 21% and CO₂ at 8%, using automated controllers. An opioid analgesic, Buprenorphine [Temgesic®] 0.01 – 0.05 mg/kg was administered postoperatively three times a day to provide adequate pain relief and reduce distress. All animals received 30 ml/kg normal saline s.c. eight hourly as fluid management.

5.3.3 ANAESTHESIA, DISSECTION AND ASSESSMENT OF INJURY
After 96 hours, surviving animals in each group were re-anaesthetised with intraperitoneal ketamine and xylazine, and underwent insertion of intravenous cannulas and tracheostomy. Anaesthesia was maintained with intravenous Saffan
infusion. Subsequently, intra-arterial access was placed in the right external carotid artery. After confirmation of depth of anaesthesia by using paw clamp, Cisatracurium besilate was administered intravenously to produce muscle relaxation. The animals were ventilated with respiratory rate of 90 breaths/min, tidal volume of 6 ml/kg, and PEEP of 2.5 cmH₂O. Animals randomised to the normocapnia group received an inspired gas mixture of 30% oxygen, and 70% nitrogen. Animals housed in environmental hypercapnia prior to surgery were ventilated with an inspired gas mixture of 5% carbon dioxide, 30% oxygen and 65% nitrogen. To minimize lung derecruitment, a recruitment manoeuvre consisting of a PEEP of 10 cmH₂O for 25 breaths was applied after initiation of mechanical ventilation and at the end of the 20 min baseline period. Body temperature was maintained at 36–37.5°C by using a thermostatically controlled blanket system and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures, and temperature were continuously measured throughout the experimental protocol. After 20 min of mechanical ventilation an arterial blood gas sample was drawn for blood gas measurement and lung static compliance determined. Subsequently, animals were ventilated for 15 minutes, arterial blood gas sample was taken and lung compliance was measured. Finally, animals were ventilated for another 15 minutes on FiO₂: 1.0 to determine alveolar arterial oxygen difference [DAaO₂]. At the end of the experiment animals were exsanguinated and blood, peritoneal fluid, bronchoalveolar lavage, and lung tissue were saved to assess injury.

5.3.4 TISSUE SAMPLING AND ASSAYS AND HISTOLOGIC AND STEREEOLOGIC ANALYSIS

Description of tissue sampling, wet dry weight ratio, BAL total protein, neutrophil and cytokine measurements and bacterial colony counts are presented in more detail in the Materials and Methods chapter [Chapter 3] under Sections 3.5. Stereological analysis is described in Section 3.5.11.

5.3.5 DETERMINATION OF THE ACTIVITY OF NF-κB PATHWAY IN LUNG TISSUE HOMOGENATES

NF-κB activity was assessed by measuring p65 DNA binding activity in the nuclear fraction of lung tissue homogenates from normocapnic and hypercapnic septic
animals. Separation of nuclear extracts from lung tissue is described in Materials and Methods chapter [Chapter 3] under Section 3.5.8. Nuclear p65 binding activity was determined using an ELISA transcription factor assay kit. More detailed description is presented in the Materials and Methods chapter [Chapter 3, Section 3.5.9.3]

5.4 STATISTICAL ANALYSIS
The distribution of data was tested for normality using the Kolmogorov-Smirnov test. Results are expressed as mean ± standard deviation [SD] for normally distributed data, and as median [interquartile range, IQR] if non-normally distributed. Data obtained at 96h were analyzed, using a Student’s t test or Mann-Whitney U test, with the Bonferroni correction as appropriate. Lung histology was analyzed by two-way analysis of variance, with group as the first factor and histologic classification [airspace, acinar tissue space, extra-acinar tissue] as the second factor. Mortality data were analyzed, using a Fisher’s exact test. A two-tailed p <0.05 was considered significant.
5.5 RESULTS

Forty animals entered to the study and all animals were randomised to receive either normocapnia [n=20] or HCA [n=20] and animal survival duration measured for up to 96h following the CLP procedure. The first eight animals from each experimental group that had survived the 96h experimental period underwent anaesthesia and assessment of the severity of the lung injury and of the inflammatory response induced by CLP.

5.5.1 SURVIVAL

Thirteen animals randomised to environmental hypercapnia survived the protocol, compared with 12 animals in the normocapnia group. Median survival time was identical in both groups [Table 5-1]. Animals in both groups lost weight to a similar extent over the course of the protocol [Table 5-1].

5.5.2 ARTERIAL CO\textsubscript{2} TENSION AND ACID BASE STATES

Arterial pH was significantly lower and arterial pCO\textsubscript{2}, bicarbonate and base deficit were significantly higher in the prolonged hypercapnia group compared with the normocapnia group, a finding compatible with persistent HCA with renal buffering [Table 5-1; Figure 5-1; Figure 5-2; Figure 5-3; Figure 5-4].

5.5.3 EFFECT ON HAEMODYNAMIC PROFILE AND LACTATE LEVELS

There was no difference in mean arterial pressure between the groups at the end of the protocol [Table 5-1]. In contrast, serum lactate was significantly lower in animals exposed to HCA compared with normocapnia [Figure 5-5].

5.5.4 EFFECT ON LUNG INJURY

PaO\textsubscript{2} was higher in animals exposed to HCA [Figure 5-6]. However, this seemed to be a physiologic effect of inhaled CO\textsubscript{2} because PaO\textsubscript{2} was not different between the groups when the animals were exposed to 100% oxygen, and there was no difference in alveolar-arterial oxygen gradient at the end of the protocol [Table 5-1]. Furthermore, there was no difference in peak Paw, elastance or static lung compliance between the groups at the end of the protocol [Table 5-1; Figure 5-7]. Lung wet/dry weight ratios were not different between the groups [Figure 5-8]. Quantitative stereological analysis demonstrated that HCA modestly but significantly reduced acinar tissue volume.
fraction and increased acinar air-space volume fraction [Figure 5-9]. These data are consistent with a small reduction in structural lung damage in animals exposed to hypercapnia [Figure 5-10].

5.5.5 EFFECT ON LUNG INFLAMMATION

HCA did not significantly alter BAL neutrophil counts compared with normocapnia [Figure 5-11]. There were no differences between the groups in BAL IL-6 or TNF-α levels, or in BAL protein concentrations compared with normocapnia [Table 5-1]. Of interest, TNF-α was not detected in the BAL in either group, and BAL IL-6 levels were much lower than that seen in the early sepsis series [Table 5-1] [131].

5.5.6 EFFECT ON LUNG NF-κB ACTIVITY

HCA decreased significantly p65 nuclear binding activity compared to normocapnia in lung nuclear extracts representing reduced NF-κB translocation and binding to DNA at 96h [Figure 5-12]. This may suggest reduced NF-κB pathway activity under hypercapnic conditions.

5.5.7 EFFECT ON PULMONARY AND SYSTEMIC BACTERIAL LOAD

There were no significant differences between the groups in the bacterial loads of the lungs, as assessed by BAL colony counts [Figure 5-15]. Similarly, there were no significant differences between the groups in the bacterial loads in the blood and peritoneal fluid at the end of the protocol [Figure 5-14; Figure 5-16]. These data confirms that CLP resulted in bacteraemia, however, prolonged hypercapnia did not alter bacterial proliferation or killing in this model.
5.6 DISCUSSION

The commonest cause of ALI/ARDS is sepsis, whether due to pneumonia or systemic infection, and is associated with the poorest outcome [8, 31]. The safety of HCA and its potential to cause adverse effects in the context of bacterial sepsis is a real concern. Both in vitro and in vivo data suggest that HCA may impair host response to invading pathogens through its anti-inflammatory/immunosuppressive action [136, 141, 302].

5.6.1 EFFECT OF HCA ON EARLY AND PROLONGED PULMONARY SEPSIS

Previously our group demonstrated that HCA protected against early and established E. coli induced pneumonia [42, 130]. In these experiments, HCA was associated with less hemodynamic alterations and less severe lung injury. Most importantly, short exposure to HCA [6 hours] did not altered bacterial load in BAL, blood and lung tissue. These findings suggested that HCA did not promote bacterial proliferation at least in the early phase of pneumonia and it blunted tissue injury. In contrast, O’Croinin et al found that prolonged hypercapnia [48h] worsened lung injury in rats and resulted in higher bacterial colony counts in lung tissue than the normocapnic controls [141]. Neutrophils isolated from hypercapnic animals had impaired phagocytic activity, indicating that HCA may have contributed to bacterial propagation and lung damage via reducing host bacterial killing capacity.

5.6.2 EFFECT OF HCA ON EARLY SYSTEMIC SEPSIS AND SEPSIS INDUCED ALI

These observations directed our attention to test the impact of hypercapnia on systemic sepsis both in the early and the later phase of sepsis. Costello and Higgins have demonstrated in a CLP model that HCA reduced the severity of early septic shock and lung injury, when the experimental time was set for 3 hours and 6 hours [131, 187]. This was reflected in the ability of HCA to slow the development of hypotension, to preserve central venous oxygen saturation and attenuate the increase in serum lactate level compared to normocapnia. In the 6 hour CLP series, animal survival was better and the time to develop septic shock was longer during HCA than in the normocapnia group. HCA also reduced the severity of lung injury and was associated with better oxygenation, lung mechanics and reduced lung permeability in both experimental series. BAL neutrophil counts were less in animals exposed to HCA,
however, there was no difference in BAL IL-6 and TNF-α levels compared to normocapnia when animals were exposed to 3 hours of HCA. More prolonged exposure to HCA [6 hours experiment] resulted in a significant decrease in BAL TNF-α levels. Importantly, BAL and peritoneal bacterial load did not differ between the normocapnia and hypercapnia group. Of note, in the 6 hours series, blood bacterial counts were less under HCA than normocapnia.

Wang et al investigated the effect of HCA on septic shock and sepsis induced lung injury in an ovine faecal peritonitis induced sepsis model [132]. The investigators used a more established sepsis model and randomised the animals to receive HCA [average FiCO₂: 0.045], dobutamine infusion or control conditions. Both HCA and dobutamine improved cardiac output and increased heart rate compared to controls and this was associated with significantly decreased serum lactate levels. Of note, HCA caused less tachycardia than dobutamine. Other cardiovascular indices, such as arterial blood pressure, pulmonary arterial pressure, systemic and pulmonary vascular resistance and left ventricular stroke work were not different between the HCA and the dobutamine groups. The cardiovascular effect of HCA and dobutamine preceded the pulmonary effects, which appeared at the later phase of sepsis [8 hours after peritonitis induction]. HCA had more protective effect in sepsis induced ALI than dobutamine, evidenced by reduced lung oedema formation, DAaO₂, shunt fraction, and serum IL-6 levels [132].

5.6.3 THE EFFECT OF CO₂ PNEUMOPERITONEUM ON ABDOMINAL SEPSIS

Intra abdominal administration of CO₂ - by means of CO₂ pneumoperitoneum - may attenuate intraabdominal sepsis induced by endotoxin [304] or CLP in various animal species [305-307]. Both pre-treatment or treatment with 30 min CO₂ laparoscopic insufflation after laparotomy and intra abdominal LPS injection significantly improved survival in the first 72h compared to control, helium, and air insufflations [308]. More recently, Metzelder et al suggested that the survival benefit of CO₂ pneumoperitoneum in CLP induced sepsis is mainly apparent at the early phase of sepsis and does not influence late mortality [i.e. death after 7 days of CLP sepsis] [305]. The protective effect of CO₂ insufflation seemed to be related to attenuated pro-
inflammatory response evidenced by decreased serum TNF-α, IL-6 levels and increased IL-10 levels. Reduced distal organ injury have also been reported in association with CO₂ pneumoperitoneum [307]. Hepatic acute phase protein gene expression was significantly reduced in CLP induced sepsis when animals were exposed to CO₂ pneumoperitoneum compared to air or helium insufflations. Others have suggested that the immunomodulatory effect of CO₂ is independent from systemic acidosis and may be related to the effect of reduced localized peritoneal pH [309].

5.6.4 EFFECT OF HCA ON PROLONGED SYSTEMIC SEPSIS INDUCED LUNG INJURY

Given the possible harmful biological effect of sustained hypercapnia on bacterial killing in pulmonary sepsis, we hypothesised that HCA would worsen prolonged systemic sepsis and sepsis induced lung injury in rats. Animals undergoing CLP were placed in an environmental chamber receiving hypercapnic gas mixture [8% CO₂] or normocapnia for 4 days. Survival at 96 hours was similar in both group, reaching 60 and 65%, respectively [normocapnia vs. HCA]. Hypotension, a common clinical symptom of sepsis and septic shock, did not develop in surviving animals and mean arterial pressure was not different between the two groups. Of note, serum lactate levels were significantly lower in the hypercapnic than the normocapnic group. In this model animals had developed moderate lung injury reflected by reduced static compliance values [0.68 ± 0.13 vs. 0.67 ± 0.04 ml/mmHg, normocapnia vs. HCA] and increased DAaO₂ [108.3 ± 28.3 vs. 95.1 ± 41.0 mmHg, normocapnia vs. HCA]. Lung physiological indices and inflammatory markers, such as neutrophil count, TNF-α, IL-6 levels were not different between animals exposed to HCA versus normocapnia. Of note, TNF-α was not detectable and IL-6 levels were very low in BAL. p65 activity in nuclear extracts from lung homogenates was significantly less in HCA animals than in normocapnic animals and this was associated with a significant, but very modest reduction in acinar tissue fraction and increased acinar airspace volume fraction under HCA. There were no differences in BAL, blood and peritoneal fluid bacterial counts after 96 hours suggesting that prolonged hypercapnia did not alter bacterial proliferation or clearance in this model.
Our findings suggest that sustained HCA is safe in the context of prolonged systemic polymicrobial sepsis. HCA did not worsen sepsis and survival and most importantly did not promote bacterial growth compared to animals under normocapnic conditions. In addition, sustained HCA moderately protected against sepsis induced lung injury and this was associated with reduced NF-κB binding activity in lung tissue, findings which are in contrast to our hypothesis and to previous observations by O’Croinin et al [141].

5.6.5 LIMITATIONS OF CURRENT WORK

There are a number of limitations to our work:

(1) In order to achieve 96 hour survival after CLP, we had to use a sepsis model which was characterised as moderate injury [See Chapter 4.0]. This meant that sepsis induced end organ injuries; including the lungs, were associated with modest changes compared to the early sepsis [131] or direct lung injury models [42]. Our group and others have also shown that the magnitude of lung injury in VILI [Chapter 6], ischaemia-reperfusion [127], and bacterial pneumonia models [42, 141] is an important factor in detection of both physiological and inflammatory changes in response to HCA. In our case, the absence of significant difference between most of the injury markers in normocapnic and hypercapnic animals could be explained at least in part by the moderate nature of the sepsis model.

(2) Prolonged hypercapnia reduced structural lung damage in CLP induced sepsis evidenced by less injurious histological changes, however the protective effect seemed to be very moderate. It is important to notice, that histological preparations were performed after BAL lavage. This may have resulted in the washout of some of the inflammatory cells and may have reduced the difference between normocapnic and hypercapnic lung tissue samples.

(3) The dynamics of disease process in sepsis is very complex. In rodents, early death after CLP occurs up to 96 hours as a consequence of tissue injury caused by overwhelming cytokine response [310]. After 96 hours, animals either recover completely or enter into a disease phase characterised with immunosuppression and late mortality [293, 294, 310]. Our study did not investigate the effect of hypercapnia after 96 hours, the actual disease stage when immunosuppression might be
predominant, and the anti-inflammatory effect of hypercapnia potentially could cause harm. Lymphocyte apoptosis, T-cell anergy, decreased T-helper$_1$/T-helper$_2$ ratio and increased IL-10/TNF-α ratio have been shown to be associated with poor outcome in septic patients [286, 301, 311]. These markers may have been more useful in investigating the effect of sustained hypercapnia in the later phase of sepsis than early pro-inflammatory cytokine levels, such as IL-6 and TNF-α.

(4) We utilised 5-8% CO$_2$ in our experiments. This produced a degree of HCA similar to that commonly observed during lung protective ventilation strategy in critically ill patients. In the context of bacterial pneumonia, 5% CO$_2$ exposure for 48h increased bacterial load in lung tissue and worsened lung injury [133]. However, this appeared to be related to attenuated neutrophil bacterial killing capacity rather than bacterial overgrowth. In contrast with this, in our experiment, similar amount of CO$_2$ [even when it was applied for more prolonged time than the bacterial pneumonia model, 96 h vs. 48 h] did not result in increased bacterial load in septic animals and was associated with moderate improvement in lung histology. The underlying mechanism of the differing results is unclear. However, it is important to notice that the mechanism by which sepsis induced ALI develops may be different in case of pulmonary and non-pulmonary origin [23, 25, 28]. In pulmonary ARDS the predominant pathology mainly involves the lung epithelium with intra-alveolar abnormalities, while extrapulmonary ARDS causes pulmonary endothelial damage featured with more diffuse interstitial oedema, larger increase in blood, but lower levels of BAL cytokines [23, 27]. In addition, exposure of the infective focus to CO$_2$ was more direct in the case of bacterial pneumonia than in CLP induced sepsis, further highlighting the importance of the source of infection.

(5) Decreased p65 DNA binding activity in lung nuclear extracts may indicate that reduced tissue injury in animals exposed to HCA was related to reduced NF-κB activity. A number of preclinical studies attest that the biological effect of HCA is attributed to the inhibition of the NF-κB pathway. Our result are in line with these observations, however, it is not clear whether the changes caused in p65 activity by HCA is ultimately good or bad in terms of prolonged sepsis induced ALI. This uncertainty partially relates to the measurement itself. Decreased global nuclear p65 levels in lung do not give
exact information on the NF-κB activity of individual cell types. Reduced NF-κB activity in epithelial cells may reduce injury or repair, while in neutrophils or macrophages it may attenuate phagocytic activity and host defence. More suitable in vitro and in vivo experiments are required to elucidate how hypercapnia modulates the activity of NF-κB pathway in prolonged sepsis.

5.7 CLINICAL IMPLICATION

Permissive hypercapnia, as part of lung protective ventilation, has been tolerated in clinical practice and has been considered to be potentially a beneficial “side effect” evidenced by large body of preclinical studies. The protective effect of permissive hypercapnia in humans, however, has not been examined directly. Kregenow et al, suggested in the secondary analysis of the ARMA trial that moderately elevated CO₂ levels may reduce mortality in the presence of more injurious ventilation strategy [69]. In addition, the lack of evidence of obvious harm of hypercapnia in clinical practice [even at extremely high levels], further strengthened the safety and application of permissive hypercapnia in critically ill patients [312].

Currently, there is no sufficient data on the impact of permissive hypercapnia in septic patients that require prolonged ventilation in the intensive care unit. As sepsis persist, most of the patients begin to exhibit signs of immune dysfunction [286, 301]. The main hallmarks of immunoparalysis are the inability to recover from the primary infection and increased susceptibility to nosocomial infections. Most importantly, the majority of deaths in sepsis occur in this phase after resuscitation. The safety of permissive hypercapnia in the context of pulmonary sepsis has been questioned based on recently emerged in vivo and in vitro experimental data [133, 135-137]. These studies suggest that hypercapnia may reduce host defence against invading pathogens when it is present for prolonged period of time. However, our study is in contrast with these findings, and suggests that, in the context of prolonged sepsis and sepsis induced ALI, hypercapnia is safe. The seemingly contradictory data from experimental studies merely indicate that more research are needed to understand how various conditions such as, disease aetiology [pulmonary vs. extrapulmonary sepsis], stage of the disease [acute versus subacute, or chronic], immune status, degree of hypercapnia, acidosis
and buffering and exposure time may modify the effect of hypercapnia in sepsis. Apart from the concern from increased infectious risk, other factors such as compromised cardiovascular, renal or cerebral function in critically ill patients might be adversely affected by permissive hypercapnia [313]. Since the biological effect of permissive hypercapnia is inherently linked to low tidal volume ventilation, it is important to investigate how hypercapnia and acidosis (or both) might influence the outcome of lung protective ventilation and disease processes in ARDS and other organs.

5.8 CONCLUSION

1. We have demonstrated that sustained hypercapnic acidosis did not worsen sepsis and attenuated sepsis induced lung injury in an in vivo rat model of CLP induced sepsis.

2. Prolonged hypercapnia reduced global p65 DNA binding activity in lung tissue suggesting that the protective effect of HCA may have been related to the inhibition of the NF-κB pathway.

3. Prolonged hypercapnia did not increase bacterial load in blood, BAL and peritoneal fluid compared to normocapnia in CLP sepsis.

4. These findings in one part reassure the safety of sustained hypercapnia in the context of prolonged sepsis, however, they do not preclude the potential harmful effect of HCA in the later stage of sepsis.
### 5.9 TABLES AND FIGURES

<table>
<thead>
<tr>
<th>Variable</th>
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<td>Median survival at 96 hours</td>
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<td>7.29 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>-3.0 ± 0.3</td>
<td>2.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td>3.5 ± 1.0</td>
<td>2.5 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak airway pressure [mmHg]</td>
<td>4.6 ± 0.3</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Static Compliance [ml/mmHg]</td>
<td>0.68 ± 0.13</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>BAL protein concentration [μg/ml]</td>
<td>159 ± 59</td>
<td>163 ± 54</td>
</tr>
<tr>
<td>BAL TNF-α [pg/ml]</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>BAL IL-6 [pg/ml]</td>
<td>0 [0,33]</td>
<td>0 [0,8]</td>
</tr>
</tbody>
</table>

**Table 5-1** Summary of physiological variables and survival in septic animals exposed to 96 hours environment Hypercapnic acidosis or Normocapnia.

<sup>a</sup>: significantly different from Normocapnia [p<0.01]

<sup>b</sup>: significantly different from Normocapnia [p<0.05]

Data are expressed as mean ± SD or median [interquartile range]. Final data are collected upon completion of experimental protocol [n=8 in each group, Normocapnia vs. HCA].
Figure 5-1 Arterial pH was significantly lower in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: p<0.01

Figure 5-2 Arterial pCO\(_2\) tension was significantly higher in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: p<0.01
Figure 5-3 Arterial HCO$_3^-$ level was significantly higher in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: $p<0.01$

Figure 5-4 Arterial BE was significantly higher in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: $p<0.01$
Arterial lactate level was significantly less in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: p<0.05

Arterial $pO_2$ tension was significantly higher in animals exposed to environmental hypercapnia [HCA] compared to Normocapnic animals surviving 96h CLP sepsis. *: p<0.05
Figure 5-7 Final lung elastance was similar between Normocapnic and hypercapnic [HCA] animals surviving 96h sepsis.

Figure 5-8 Lung wet/dry weight ratio, representing lung oedema formation, did not differ between surviving animals exposed to Normocapnia vs. Hypercapnic acidosis [HCA].
Figure 5-9 Histogram representing stereological assessment of the extent of histologic injury in prolonged sepsis in Normocapnic and Hypercapnic animals [HCA]. *: significantly different from normocapnia, p< 0.05.

Figure 5-10 Representative lung histological pictures from normocapnic and hypercapnic [HCA] animals after 96 hours CLP induced sepsis. Animals exposed to HCA had more preserved lung structure and less cellular infiltration than normocapnic animals.
Figure 5-11 Histogram representing mean BAL neutrophil count in normocapnic and hypercapnic animals undergoing CLP induced sepsis.

Figure 5-12 Nuclear p65 activity was significantly reduced under hypercapnic [HCA] than in normocapnic condition in septic animals. *: p<0.05
Figure 5-13 Histogram representing bacterial colony forming units [CFUs] in blood, BAL and peritoneal fluid in animals surviving prolonged sepsis. There was no difference in CFUs under normocapnia compared to hypercapnia.

Figure 5-14 Box plots of bacterial count in blood from Normocapnic and Hypercapnic animals [HCA].
Figure 5-15 Box plots of bacterial count in BAL from Normocapnic and Hypercapnic animals [HCA].

Figure 5-16 Box plots of bacterial count in peritoneal fluid from Normocapnic and Hypercapnic animals [HCA].
CHAPTER 6: DEVELOPMENT OF AN IN VIVO VENTILATION INDUCED LUNG INJURY MODEL

6.1 ABSTRACT

Background: Pre-clinical models of ventilation induced ALI are well described in the literature. However, the precise impact of factors such as animal age/weight, animal batch, and of alterations in ventilation parameters such as inspiratory pressure and respiratory rate are not clear. We wished to evaluate the impact of these factors on injury severity and utilize these insights to develop reproducible models of moderate and severe VILI.

Methods: Adult, male Sprague-Dawley rats were utilised in all studies. Using an iterative design approach, we examined the impact of: (1) animal age/weight [<375g versus >425g]; (2) applied inspiratory pressure [25, 27.5 and 30 cmH\textsubscript{2}O]; (3) respiratory rate [15 versus 20 breaths/minute]; and (4) batch-to-batch animal variability. We then utilized these insights to analyze the evolution of VILI over 6h and to produce a moderate and a severe VILI ventilation strategy. We also determined the extent of injury produced with each ventilation strategy.

Results: Younger animals withstand injurious ventilation better than older animals. This is evidenced by better oxygenation, static compliance and lower alveolar neutrophil counts in the younger animal group. Increasing PIP increases mortality and worsens lung injury. Threshold pressure at which lung injury starts to accelerate is above 27.5 cmH\textsubscript{2}O PIP. Both time and respiratory rate affected injury severity, with 4h the best time period and respiratory rates of 15-18 producing injury while maintaining normocapnia. Respiratory rate proved to be the most effective method to vary the degree of lung injury. Increasing the respiratory rate from 15 to 18 breaths/min resulted in a much more severe injury, and a reduced animal survival. Differing animal batches can respond differently to the same injurious ventilation strategy.

Conclusion: Multiple factors, both animal specific [age/weight and animal batch] and ventilation strategy specific [inspiratory pressure, respiratory rate, time] alter the severity of stretch induced lung injury. We determined two mechanical ventilation settings for our further experiments. (1) Moderate VILI: PIP: 30 cmH\textsubscript{2}O and RR: 15
breaths/min and; (2) *more severe* VILI: PIP: 30 cmH$_2$O and RR: 18 breaths/min for 4h. All future experiments were limited to animals from the same batch and to animals with a body weight [BW] of less than 400 g in order to reduce variability.
6.2 INTRODUCTION

Relevant and comparable animal models are essential in research. According to the most recent recommendations by the American Thoracic Society [ATS], animal models of ALI/ARDS should capture the essential pathological features of acute lung injury that are seen in humans [314]. These include: (1) histological evidence of tissue injury; (2) alteration of the alveolar capillary membrane; (3) inflammatory response; (4) evidence of physiological dysfunction. The working group also defined measurements that can be considered “very relevant” or “somewhat relevant” in order to achieve wider comparability among scientific studies [314]. Although our model was developed before the ATS guideline, our aim was to generate a model where the above mentioned four features of ALI are present. In addition, most of the measurements used in our final experiments fulfilled the criteria of the “very relevant” category suggested by the ATS. We also had to consider that multiple factors, such as animal species [84, 315]; age of the animals [271-273], applied pressure/volume values [119, 316], length of injurious [115] ventilation, and batch to batch variability may influence the magnitude of ALI.

We have used a rodent VILI model. The core of the problem to develop a suitable VILI model in this species is related to anatomical factors [See Chapter 3, Section 3.3]. Rodents are more susceptible to excessive mechanical stretch induced injury [50, 51, 114, 115] than large species or humans [116-119]. The rapid onset of pulmonary edema leaves shorter time to recruit neutrophils into the lung tissue and may result in early mortality. To find the balance between survival and injury severity that features the main characteristics of ALI/VILI is challenging and needs extensive pilot work. Our arguments in favour for using a rat model of VILI were: (1) the relative ease of performing surgery, physiological measurements and repetitive blood sampling without major impact on the cardiovascular system; (2) the fact that the time frame of injurious ventilation that result in obvious mechanical injury and inflammatory response is reasonable, ranging from 2-8 hours; (3) the knowledge that the rodent model of VILI is well characterized in the literature and has been used widely to investigate the pathophysiology of VILI; and (4) the extensive experience that our group has in various in vivo rat ALI models[42, 125, 127, 128, 130, 131, 187, 233].
6.3 METHODS

All experiments were carried out under a licence from the Department of Health and Children, Ireland, and following approval from the Institutional Animal Care Research Ethics Committee of the National University of Ireland, Galway. Using an iterative design approach, we examined the impact of: (1) animal age/weight, (2) applied inspiratory pressure, (3) respiratory rate, and (5) impact of batch on VILI. We also looked at injury dynamics and compared injury severity in moderate and severe VILI. All data were continuously analysed and experimental protocols modified over time based on the observations arising from previous experiments. We excluded all experiments from the analysis that did not fulfil the inclusion criteria, experiments with technical problem, or with incomplete physiological measurements.

6.3.1 ANIMAL SURGERY

Adult male Sprague-Dawley rats were utilized. Anesthesia was induced with intraperitoneal ketamine and xylazine. After confirming depth of anesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein and further anesthesia maintained with an intravenous Saffan infusion. A tracheostomy tube was inserted, secured and intra-arterial access was placed in the right carotid artery. Following confirmation of depth of anesthesia using paw clamp, Cisatracurium besilate was administered intravenously to produce muscle relaxation. The animals were ventilated with an inspired gas mixture of FiO₂ of 0.3, respiratory rate of 90 breaths/min, tidal volume of 6 ml/kg, and positive end-expiratory pressure of 2.5 cmH₂O. To minimize lung de-recruitment, a recruitment maneuver consisting of a PEEP of 10 cmH₂O for 25 breaths was applied. Body temperature was maintained at 36-37.5°C and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures and temperature were continuously measured throughout the experimental protocol. After 20 minutes an arterial blood gas sample was drawn for blood gas measurement and lung compliance measured in order to confirm baseline stability. Subsequently animals were exposed to dedicated injury protocols.
6.3.2 INJURY PROTOCOLS APPLIED IN THE PILOT SERIES

Overall, experiments with four distinct ventilator settings generating different degrees of mechanical injury were included. Time course of injury was 6 hours except in the last experimental series, which was 4 hours in duration [[Table 6-1]. From a total number of 52 animals undergoing 6 hours VILI experiments, 38 were included and 14 animals were excluded. Exclusion was due to abnormal baseline parameters [hypoxaemia, hypotension], animal death during surgery [missed arterial cannulation acute haemorrhage, sudden death], other than 600 ml/min airflow setting on the ventilator or incomplete physiological measurements[Table 6-1]. Ventilation induced lung injury protocols included in the analysis of pilot data.

<table>
<thead>
<tr>
<th>Peak Inspiratory Pressure [cmH₂O]</th>
<th>Respiratory Rate [breaths/minute]</th>
<th>Time [hours]</th>
<th>Total number [n]</th>
<th>Included [n]</th>
<th>Excluded [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>20</td>
<td>6</td>
<td>11</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>27.5</td>
<td>15</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>6</td>
<td>16</td>
<td>11</td>
<td>5</td>
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<tr>
<td>30</td>
<td>20</td>
<td>6</td>
<td>17</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>

6.3.2.1 Series I. Effect of age on the dynamics of ventilation induced lung injury.

As the animals get standard amounts of caloric intake, the major determinant of weight is animal age. We defined older animals as body weight [BW] over 425 g and younger animals as less than 375 g. This represented around 4 weeks of age difference between the two groups based on a growth rate chart for male Sprague-Dawley rats provided by Charles River Laboratories International [371]. Animals were ventilated with peak inspiratory pressure [PIP]: 25cmH₂O, respiratory rate [RR]: 20/min, and FiO₂: 0.3 for 6 hours.

6.3.2.2 Series II. Effect of peak inspiratory pressure on the development of VILI.

The aim of this analysis was to determine a PIP that results in marked changes in lung injury markers while maintaining animal survival at an acceptable level. In order to eliminate the effect of age on injury dynamics, we included rats with similar BW. Applied PIP and RR were the following: 25cmH₂O and 20/min [25/20], 27.5 cmH₂O and
15/min [27.5/15] 30 cmH₂O and 15/min [30/15]. The different respiratory rates were necessary to maintain similar PaCO₂ ranges.

6.3.2.3 Series III. Effect of differing respiratory rates on VILI

We compared the effect of a RR of 15/min with RR: 20/min in rats receiving mechanical ventilation with equal PIP [30cmH₂O] for 6 hours.

6.3.2.4 Series IV. Impact of differing animal batches on VILI

In this analysis, we looked at the impact of different animal batches on lung injury development in two ventilator protocols. We compared April 2008 and May 2008 batches in rats subjected to PIP: 30 cmH₂O and RR: 15/min and September 2008 and October 2008 batches in rats subjected to PIP: 30 cmH₂O and RR: 20/min.

6.3.2.5 Series V. Evaluation of the dynamics of VILI over time.

Our aim was to define a time point at which a significant injury was produced by where animal survival remained reasonable using a setting PIP: 30cmH₂O and RR: 20/min.

6.3.2.6 Series VI. Description of final ventilation induced lung injury models. Moderate versus severe VILI.

We compared the lung injury pattern in our final in vivo rat VILI models. Animals ventilated with PIP: 30cmH₂O, RR: 15/min were defined as moderate VILI and animals subjected to PIP:30 cmH₂O, RR: 20/min were defined as severe VILI.

6.4 DATA ANALYSIS

Data are expressed as mean +/- SD and were analysed retrospectively. The purpose of these experiments was to examine the factors that contribute to injury severity in the VILI model. Therefore, the same animals were included more than one Series. Some of the animals ventilated with 25/20 protocol in Series I were included in Series II, and animals ventilated with 30/15 protocol in Series II were included in Series III. –IVa. Animals ventilated with the 30/20 protocol were included in Series III, IVb, V. No specific hypotheses were tested, and the analysis is therefore descriptive.
6.4.1 RESULTS

6.4.1.1 Series I. Effect of age on the dynamics of ventilation induced lung injury.

We included nine animals from the 25/20 injury protocol group in this analysis. The average BW of the older animal group [n=6] was 464 ± 12 g, and of the younger animal group 343 ± 6 g [n=3]. There were no differences between the groups at baseline with regard to PaO₂, PaCO₂, arterial pH, serum lactate and bicarbonate, and static compliance mean arterial pressure [MAP] was higher in the younger animal group at baseline and throughout the experiment [Table 6-2].
<table>
<thead>
<tr>
<th>Variable</th>
<th>BW&lt;400g [PIP/RR:25/20]</th>
<th>BW&gt;400g [PIP/RR:25/20]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Animal Weight [g]</td>
<td>343 ± 6</td>
<td>464 ± 12</td>
</tr>
<tr>
<td>Animal survival at 6 h [%]</td>
<td>3/3 [100%]</td>
<td>2/6 [33%]</td>
</tr>
<tr>
<td>Duration of survival [minutes]</td>
<td>360 ± 0</td>
<td>310 ± 48</td>
</tr>
<tr>
<td>Mean Arterial Pressure [mmHg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>154 ± 11</td>
<td>119 ± 57</td>
</tr>
<tr>
<td>1 Hours</td>
<td>131 ± 14</td>
<td>103 ± 35</td>
</tr>
<tr>
<td>2 Hours</td>
<td>130 ± 15</td>
<td>109 ± 21</td>
</tr>
<tr>
<td>3 Hours</td>
<td>113 ± 26</td>
<td>101 ± 19</td>
</tr>
<tr>
<td>4 Hours</td>
<td>111 ± 22</td>
<td>113 ± 12</td>
</tr>
<tr>
<td>5 Hours</td>
<td>102 ± 22</td>
<td>95 ± 18</td>
</tr>
<tr>
<td>6 Hours</td>
<td>105 ± 30</td>
<td>101 ± 23</td>
</tr>
<tr>
<td>Arterial $O_2$ tension [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>19.0 ± 0.8</td>
<td>18.6 ± 0.8</td>
</tr>
<tr>
<td>Final</td>
<td>17.5 ± 0.8</td>
<td>10.4 ± 4.5</td>
</tr>
<tr>
<td>Arterial $CO_2$ tension [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.7 ± 0.7</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Final</td>
<td>4.7 ± 0.8</td>
<td>5.9 ± 1.7</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.44 ± 0.05</td>
<td>7.43 ± 0.20</td>
</tr>
<tr>
<td>Final</td>
<td>7.36 ± 0.02</td>
<td>7.25 ± 0.18</td>
</tr>
<tr>
<td>Serum Bicarbonate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>21.1 ± 0.58</td>
<td>22.0 ± 0.8</td>
</tr>
<tr>
<td>Final</td>
<td>20.8 ± 2.6</td>
<td>17.9 ± 4.9</td>
</tr>
<tr>
<td>Base Excess [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-5.2 ± 1.0</td>
<td>-3.9 ± 0.9</td>
</tr>
<tr>
<td>Final</td>
<td>-4.8 ± 3.7</td>
<td>-7.5 ± 5.6</td>
</tr>
<tr>
<td>Serum Lactate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.4 ± 0.5</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>Final</td>
<td>2.8 ± 1.8</td>
<td>4.6 ± 3.5</td>
</tr>
<tr>
<td>Static Compliance [ml/cmH₂O]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.04 ± 0.06</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>Final</td>
<td>0.76 ± 0.09</td>
<td>0.66 ± 0.17</td>
</tr>
<tr>
<td>Compliance change [%]</td>
<td>27 ± 7</td>
<td>37 ± 17</td>
</tr>
<tr>
<td>BAL neutrophil count [cell/ml]</td>
<td>14 140 ± 8063 [n=3]</td>
<td>36 480± 19007 [n=2]</td>
</tr>
</tbody>
</table>

Table 6-2 Summary of survival, baseline and final physiological parameters in young [BW<375 g] and old [BW>425 g] animals. Protocol: PIP: 25 cmH₂O, RR: 20 breaths/min, experimental time: 6h.
6.4.1.1.1 Survival

All younger animals survived the 6h experimental period while the older animal group had a sudden increase of mortality after 4h mechanical ventilation and only 33% [2/6] of the animals were alive at the end of the experiment [Figure 6-1]. Mean survival time was more prolonged in the younger animal group than in the older animal group, 360 ± 0 vs. 310 ± 48 min, respectively [Table 6-2].

![Figure 6-1 Survival time in young [BW < 375 g] and old animals [BW > 425g] ventilated with PIP: 25 cmH₂O and RR: 20/min.](image)

6.4.1.1.2 Arterial pCO₂ and acid base

The older animal group had much rapid increase of paCO₂ levels than the younger animal group, and this was associated with more gradual decrease in pH levels and more severe lactic acidosis [Figure 6-2; Figure 6-3; Table 6-2]. Both serum bicarbonate levels and base excess were lower in the older animal group compared to the younger animal group [Table 6-2].
Figure 6-2 Arterial CO₂ tension over time in young [BW<375g] and old [BW>425g] animals. Protocol: PIP: 25 cmH₂O, RR: 20/min, experimental time: 6 h.

Figure 6-3 Arterial pH over time in young [BW<374g] and old [BW>425g] animals. Protocol: PIP: 25 cmH₂O, RR: 20/min, experimental time: 6 h.
6.4.1.1.3 Lung injury

Arterial pO$_2$ tension was lower at each time points after 1 hour of injurious ventilation in the older animal group than the younger animal group [Figure 6-4]. Static compliance decreased by 37% in the older animal group; and by 27% in the younger animal group by the end of the experiment [Table 6-2; Figure 6-5]. Final lung elastance was also higher in the older animal group compared to the younger animal group [Figure 6-6]. This was associated with higher neutrophil count in the BAL samples from the older animal group [Table 6-2].

![Figure 6-4](image-url)

**Figure 6-4** Arterial O$_2$ tension over time in young [BW<375g] and old [BW> 425 g] animals. Protocol: PIP: 25 cmH$_2$O, RR: 20/min, experimental time: 6 h.
Figure 6-5 Static compliance over time in young [BW<375g] and old [BW>425 g] animals. Protocol: PIP: 25 cmH₂O, RR: 20/min, experimental time: 6 h.

Figure 6-6 Final elastance in young [BW<375g] and old [BW>425g] animals. Protocol: PIP: 25 cmH₂O, RR: 20/min, experimental time: 6 h.
6.4.1.2 Series II. Effect of peak inspiratory pressure on the severity of ventilation induced lung injury

We included 12 animals in this series. Since age has impact on injury development, animals less than <400 g at around the same weight range were selected for the analysis. There were no large differences between the groups at baseline with regard to MAP, PaO₂, PaCO₂, arterial pH, serum lactate and bicarbonate, and static compliance. The average weight was 343±6 g in the 25/20, 361±22 g in the 27.5/15 group and 358±22 g in the 30/15 group [Table 6-3].
<table>
<thead>
<tr>
<th>Variable</th>
<th>25/20</th>
<th>27.5/15</th>
<th>30/15</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>[PIP/RR]</td>
<td>[PIP/RR]</td>
<td>[PIP/RR]</td>
</tr>
<tr>
<td>Number of animals</td>
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<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Animal Weight [g]</td>
<td>343 ± 6</td>
<td>361 ± 22</td>
<td>358 ± 22</td>
</tr>
<tr>
<td>Mean survival time [min]</td>
<td>360</td>
<td>360</td>
<td>208 ± 36</td>
</tr>
<tr>
<td>Survival at 6 hours</td>
<td>100 % [3/3]</td>
<td>100 % [5/5]</td>
<td>0 % [0/4]</td>
</tr>
<tr>
<td>Mean arterial pressure [mmHg]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>154 ± 11</td>
<td>160 ± 12</td>
<td>160 ± 17</td>
</tr>
<tr>
<td>1 Hours</td>
<td>131 ± 14</td>
<td>136 ± 7</td>
<td>134 ± 7</td>
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<tr>
<td>2 Hours</td>
<td>130 ± 15</td>
<td>137 ± 12</td>
<td>136 ± 24</td>
</tr>
<tr>
<td>3 Hours</td>
<td>113 ± 26</td>
<td>132 ±15</td>
<td>128 ± 20</td>
</tr>
<tr>
<td>4 Hours</td>
<td>111 ± 22</td>
<td>128 ±14</td>
<td>137</td>
</tr>
<tr>
<td>5 Hours</td>
<td>102 ± 22</td>
<td>120 ± 10</td>
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<tr>
<td>6 Hours</td>
<td>105 ± 30</td>
<td>127 ± 10</td>
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<tr>
<td>Arterial O₂ tension [kPa]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>19.0 ± 0.8</td>
<td>18.0 ± 1.3</td>
<td>18.0 ± 0.8</td>
</tr>
<tr>
<td>1 Hours</td>
<td>19.0 ± 0.6</td>
<td>17.7 ± 1.6</td>
<td>18.1 ± 3.4</td>
</tr>
<tr>
<td>2 Hours</td>
<td>18.9 ± 1.0</td>
<td>17.2 ± 1.8</td>
<td>15.4 ± 1.9</td>
</tr>
<tr>
<td>3 Hours</td>
<td>19.0 ± 0.8</td>
<td>16.7 ± 1.8</td>
<td>7.0 ± 2.03</td>
</tr>
<tr>
<td>4 Hours</td>
<td>17.3 ± 1.5</td>
<td>16.4 ± 1.0</td>
<td>9.4</td>
</tr>
<tr>
<td>5 Hours</td>
<td>18.2 ± 0.3</td>
<td>15.7 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>6 Hours</td>
<td>17.5 ± 0.8</td>
<td>14.9 ± 1.3</td>
<td>-</td>
</tr>
<tr>
<td>Arterial CO₂ tension [kPa]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.7 ± 0.7</td>
<td>4.2 ± 0.4</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>1 Hours</td>
<td>3.5 ± 0.3</td>
<td>4.7 ± 0.9</td>
<td>5.3 ± 3.0</td>
</tr>
<tr>
<td>2 Hours</td>
<td>3.7 ± 0.4</td>
<td>4.9 ± 1.3</td>
<td>5.8 ± 3.1</td>
</tr>
<tr>
<td>3 Hours</td>
<td>3.8 ± 0.4</td>
<td>5.1 ± 1.1</td>
<td>6.8 ± 2.2</td>
</tr>
<tr>
<td>4 Hours</td>
<td>4.2 ± 0.8</td>
<td>5.4 ± 0.8</td>
<td>7.2</td>
</tr>
<tr>
<td>5 Hours</td>
<td>4.0 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>6 Hours</td>
<td>4.7 ± 0.8</td>
<td>5.7 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.44 ± 0.05</td>
<td>7.46 ± 0.03</td>
<td>7.46 ± 0.05</td>
</tr>
<tr>
<td>1 Hours</td>
<td>7.44 ± 0.02</td>
<td>7.44 ± 0.06</td>
<td>7.39 ± 0.15</td>
</tr>
<tr>
<td>2 Hours</td>
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<td>7.35 ± 0.13</td>
</tr>
<tr>
<td>3 Hours</td>
<td>7.42 ± 0.01</td>
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<td>7.20 ± 0.14</td>
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<td>7.40 ± 0.05</td>
<td>7.39 ± 0.04</td>
<td>6.97</td>
</tr>
<tr>
<td>5 Hours</td>
<td>7.42 ± 0.05</td>
<td>7.39 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>6 Hours</td>
<td>7.36 ± 0.02</td>
<td>7.36 ± 0.04</td>
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<tr>
<td>Serum Bicarbonate [mmol/l]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>21.1 ± 0.6</td>
<td>24.6 ± 1.0</td>
<td>22.5 ± 1.8</td>
</tr>
<tr>
<td>Final</td>
<td>20.8 ± 2.6</td>
<td>23.4 ± 1.9</td>
<td>15.3 ± 4.7</td>
</tr>
<tr>
<td>Base Excess [mmol/l]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-5.2 ± 1.0</td>
<td>-0.7 ± 1.2</td>
<td>-3.7 ± 2.1</td>
</tr>
<tr>
<td>Final</td>
<td>-4.8 ± 3.7</td>
<td>-0.9 ± 2.34</td>
<td>-9.6 ± 6.5</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.4 ± 0.5</td>
<td>2.8 ± 0.5</td>
<td>1.4 ± 1.4</td>
</tr>
<tr>
<td>Final</td>
<td>2.8 ± 1.8</td>
<td>2.2 ± 0.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Static Lung compliance [ml/cmH₂O]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.04 ± 0.06</td>
<td>0.99 ± 0.31</td>
<td>1.29 ± 0.58</td>
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<tr>
<td>Final</td>
<td>0.76 ± 0.09</td>
<td>0.73 ± 0.13</td>
<td>0.63 ± 0.33</td>
</tr>
<tr>
<td>Compliance change [%]</td>
<td>27 ± 7</td>
<td>21 ± 23</td>
<td>38 ± 29</td>
</tr>
</tbody>
</table>

Table 6-3 Analysis of threshold phenomena in animals ventilated with increasing peak inspiratory pressure, PIP: 25, 27.5, 30 cmH₂O. Experimental time: 6h.
6.4.1.2.1 Survival

All animals survived the 6h injurious ventilation in the 25/20 and 27.5/15 groups. In contrast with this, none of the animals subjected to 30/15 ventilation protocols were alive at 5 and 6h. In addition, survival rate was only 55% at 4 hours and mean survival time 208 ± 36 min in the 30/15 group [Figure 6-7; Table 6-3].

![Figure 6-7](image.png)

**Figure 6-7** Survival over time in animals ventilated with increasing peak inspiratory pressure; PIP: 25, 27.5, 30 cmH₂O.

6.4.1.2.2 Arterial pCO₂ and acid base balance

Arterial CO₂ tension was marginally higher in the 27.5/15 group compared to 25/20 groups and remained steady over the 6h experimental protocol. However, in the 30/15 group, paCO₂ increased gradually over time – reflecting the severity of the lung injury - and was much higher at each time point than in the 25/20 and 27.5/15 groups [Figure 6-8]. Arterial pH did not change markedly over time in the 25/20 and 27.5/15 groups compared to 30/15 group [Figure 6-9]. Both final bicarbonate and base excess levels were worst in the 30/15 animal group and this was associated with higher serum lactate levels [Table 6-3].
Figure 6-8 Arterial CO$_2$ tension [pCO$_2$] over time in animals ventilated with increasing peak inspiratory pressure; PIP: 25, 27.5, 30 cmH$_2$O.

Figure 6-9 Arterial pH over time in animals ventilated with increasing peak inspiratory pressure; PIP: 25, 27.5, 30 cmH$_2$O.
6.4.1.2.3 Lung injury

Arterial pO₂ tension did not vary markedly over time in the 25/20 and 27.5/15 groups [Figure 6-10; Table 6-3]. However, final arterial O₂ tension was less with increasing PIP [Table 6-3]. Arterial oxygenation in animals receiving FiO₂: 1.0 at the end of the experiment was worst in the 30/15 group [Figure 6-11]. Static lung compliance decreased in each group after mechanical ventilation however, the largest drop in compliance compared to baseline values was seen in the 30/15 group reaching 38% [Table 6-3; Figure 6-12]. Final elastance was highest in the 30/15 group, and was similar in the 25/20 and 27.5/15 groups [Figure 6-13].

Figure 6-10 Arterial O₂ tension [pO₂] over time in animals ventilated with increasing peak inspiratory pressure; PIP: 25, 27.5, 30 cmH₂O.
Figure 6-11 Arterial O$_2$ tension in animals receiving FiO$_2$: 1.0. Peak inspiratory pressure: 25, 27.5, 30 cmH$_2$O, arterial O$_2$ tension: 72.3 ± 2.0, 67.0 ± 6.4, 10.6 ± 5.6 kPa, respectively.

Figure 6-12 Evolution of lung static compliance over time in animals ventilated with increasing peak inspiratory pressure, PIP: 25, 27.5, 30 cmH$_2$O.
6.4.1.3 Series III. Effect of respiratory rate on the severity of ventilation induced lung injury.

We included 24 animals in this series and compared the effect of increasing respiratory rate on the degree of lung injury. Animals were receiving either RR: 15/min or 20/min with the same PIP [30 cmH₂O]. Both animal groups had a BW less than 400 g. MAP was higher at baseline in the 30/15 group than the 30/20 group. However, MAP dropped by 32% over 6 hours in the 30/20 group compared to 27% in the 30/15 group. There were no large differences between the groups at baseline with regard to \( \text{paO}_2 \), serum lactate and bicarbonate, and static compliance. Arterial CO₂ tension was lower and pH was higher in the 30/15 group at baseline than in the 30/20 group [Table 6-4].
<table>
<thead>
<tr>
<th>Variable</th>
<th>30/15 [PIP/RR]</th>
<th>30/20 [PIP/RR]</th>
</tr>
</thead>
<tbody>
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<td>Number of animals</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Animal Weight [g]</td>
<td>397 ± 37</td>
<td>361 ± 20</td>
</tr>
<tr>
<td>Mean survival time [min]</td>
<td>250 ± 72</td>
<td>200 ± 96</td>
</tr>
<tr>
<td>Survival at 6 h [%]</td>
<td>2/11 [18%]</td>
<td>2/13 [15%]</td>
</tr>
<tr>
<td>Mean arterial pressure [mmHg]</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>171 ± 15</td>
<td>148 ± 21</td>
</tr>
<tr>
<td>1 Hours</td>
<td>141 ± 11</td>
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<td>2 Hours</td>
<td>140 ± 18</td>
<td>108 ± 19</td>
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<td>3 Hours</td>
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<td>119 ± 23</td>
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<td>4 Hours</td>
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<td>5 Hours</td>
<td>125 ± 8</td>
<td>99 ± 19</td>
</tr>
<tr>
<td>6 Hours</td>
<td>125 ± 8</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>Arterial O\textsubscript{2} tension [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>18.3 ± 0.8</td>
<td>18.8 ± 0.8</td>
</tr>
<tr>
<td>1 Hours</td>
<td>18.3 ± 2.0</td>
<td>16.1 ± 3.5</td>
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<tr>
<td>2 Hours</td>
<td>15.9 ± 1.9</td>
<td>13.9 ± 4.2</td>
</tr>
<tr>
<td>3 Hours</td>
<td>9.3 ± 5.0</td>
<td>11.9 ± 4.6</td>
</tr>
<tr>
<td>4 Hours</td>
<td>7.8 ± 5.0</td>
<td>11.2 ± 4.7</td>
</tr>
<tr>
<td>5 Hours</td>
<td>7.1 ± 4.6</td>
<td>11.0 ± 4.5</td>
</tr>
<tr>
<td>6 Hours</td>
<td>6.7 ± 3.9</td>
<td>10.7 ± 4.3</td>
</tr>
<tr>
<td>Arterial O\textsubscript{2} tension on FiO\textsubscript{2}: 1.0 [kPa]</td>
<td>18.3 ± 19.7</td>
<td>30.6 ± 23.1</td>
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<tr>
<td>Arterial CO\textsubscript{2} tension [kPa]</td>
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</tr>
<tr>
<td>Baseline</td>
<td>3.9 ± 0.5</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>1 Hours</td>
<td>4.3 ± 1.8</td>
<td>3.4 ± 0.7</td>
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<tr>
<td>2 Hours</td>
<td>4.8 ± 1.9</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>3 Hours</td>
<td>5.4 ± 1.8</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>4 Hours</td>
<td>5.8 ± 1.7</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>5 Hours</td>
<td>6.2 ± 1.7</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>6 Hours</td>
<td>6.3 ± 1.7</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>Arterial pH</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.46 ± 0.04</td>
<td>7.42 ± 0.04</td>
</tr>
<tr>
<td>1 Hours</td>
<td>7.44 ± 0.09</td>
<td>7.45 ± 0.05</td>
</tr>
<tr>
<td>2 Hours</td>
<td>7.41 ± 0.10</td>
<td>7.36 ± 0.11</td>
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<tr>
<td>3 Hours</td>
<td>7.28 ± 0.19</td>
<td>7.29 ± 0.16</td>
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<tr>
<td>4 Hours</td>
<td>7.24 ± 0.19</td>
<td>7.27 ± 0.18</td>
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<td>5 Hours</td>
<td>7.22 ± 0.18</td>
<td>7.26 ± 0.18</td>
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<tr>
<td>6 Hours</td>
<td>7.21 ± 0.18</td>
<td>7.26 ± 0.18</td>
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<tr>
<td>Serum Bicarbonate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.5 ± 1.7</td>
<td>23.1 ± 0.94</td>
</tr>
<tr>
<td>Final</td>
<td>16.0 ± 5.6</td>
<td>16.7 ± 6.2</td>
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<tr>
<td>Base Excess [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-2.2 ± 2.1</td>
<td>-2.3 ± 1.3</td>
</tr>
<tr>
<td>Final</td>
<td>-9.1 ± 6.6</td>
<td>-10.4 ± 7.2</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.9 ± 1.2</td>
<td>2.1 ± 0.4</td>
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<tr>
<td>Final</td>
<td>9.0 ± 12.6</td>
<td>8.1 ± 6.2</td>
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<td>Static Compliance [ml/mmHg]</td>
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<tr>
<td>Baseline</td>
<td>1.14 ± 0.36</td>
<td>1.0 ± 0.14</td>
</tr>
<tr>
<td>Final</td>
<td>0.89 ± 0.48</td>
<td>0.62 ± 0.16</td>
</tr>
<tr>
<td>Compliance change [%]</td>
<td>29 ± 21</td>
<td>38 ± 12</td>
</tr>
<tr>
<td>BAL neutrophil count [cell/ml]</td>
<td></td>
<td></td>
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<tr>
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<td>21478 ± 12092</td>
<td>27513 ± 21678</td>
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<tr>
<td>[n=8]</td>
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<tr>
<td>[n=10]</td>
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</table>
Table 6-4 Effect of respiratory rate on physiological parameters and survival in rats ventilated with PIP: 30 cmH₂O and RR: 15 v. 20/minute.

6.4.1.3.1 Survival

Mean survival time was shorter when the animals were ventilated with RR: 20/min [200 ± 96 vs. 250 ± 72 min, RR: 20 vs. RR: 15; Table 6-4]. Animal survival started to decline after 3h mechanical ventilation in the 30/15 group as opposed to 1h in the 30/20 group [Figure 6-14].

Figure 6-14 Effect of respiratory rate on survival rate over time in rats ventilated with PIP: 30 cmH₂O and RR: 15 vs. 20/min.

6.4.1.3.2 Arterial pCO₂ and acid base

Animals receiving RR: 15/min had higher paCO₂ and lower pH at each time point. Final serum bicarbonate, base excess and lactate levels did not differ markedly between the two groups [Table 6-4].
6.4.1.3.3 Lung injury

Arterial pO₂ tension was initially worst in the group ventilated with RR: 20/min, however, at 4, 5, 6h oxygenation was less in the RR: 15 group. Arterial oxygenation in animals receiving FiO₂: 1.0 at the end of the experiment was higher in the 30/20 group compared to the 30/15 group [30 ± 23.1 vs. 18.3 ± 19.7 kPa, respectively]. Static lung compliance decreased in each group after injurious ventilation. Reduction of compliance was 29% in the 30/15 group and 38% in the 30/20 group [Table 6-4]. BAL neutrophil count did not differ greatly between the two groups [Table 6-4].

6.4.1.4 Series IV. Impact of differing animal batches on the severity of ventilation induced lung injury.

Two sets of animal groups were included in this series. Rats ventilated with PIP: 30 cmH₂O and RR: 15/min and PIP: 30 cmH₂O and RR: 20/min. Within each group we compared the dynamics of lung injury between two batches of animals.

Series III-a: March batch vs. April batch. PIP: 30 cmH₂O, RR: 15/min [Table 6-5].

Series III-b: September batch vs. October batch. PIP: 30 cmH₂O, RR: 20/min [Table 6-6].

6.4.1.4.1 Series III-a: April versus March animal batch

Baseline parameters did not differ between the two groups.

6.4.1.4.2 Survival

March batch had more prolonged survival than the April batch [279 ± 69 vs. 234 ± 74 min]. Changes in MAP throughout the experiments were similar in both batches [Table 6-5; Figure 6-15].
Table 6-5 Demonstration of variability between two batches of animals [March vs. April] ventilated with the same injurious ventilation. Peak inspiratory pressure [PIP]: 30cmH₂O, respiratory rate [RR]: 15/minute.
Figure 6-15 Survival over time in animals from March vs. April batch with the same ventilator setting [PIP: 30 cmH₂O; RR: 15/min].

6.4.1.4.3 Arterial pCO₂ and acid base

There were no major difference in paCO₂ tension, arterial pH, serum bicarbonate and base excess after injurious ventilation between March vs. April batches [Table 6-5].

6.4.1.4.4 Lung injury

Arterial pO₂ tension decreased in a similar fashion in the two groups, however, final oxygenation on FiO₂: 1.0 was worst in the April batch than the March batch [Figure 6-16]. Contrary, static compliance was worst in the March batch and reduced by 32% from baseline compared to the April-08 batch [11%] [Table 6-5; Figure 6-17].
Figure 6-16 Final arterial O₂ tension in animals receiving FiO₂: 1.0. March vs. April batch [20.8 ± 22.6 vs. 16.2 ± 19.6 kPa] with the same ventilator setting [PIP: 30 cmH₂O; RR: 15/min].

Figure 6-17 Evolution of lung static compliance over time in animals from March vs. April batch with the same ventilator setting [PIP: 30cmH₂O; RR: 15/min].
6.4.1.4.5 Series III-b: September versus October animal batch

Baseline parameters did not differ significantly between the two groups [Table 6-6].

6.4.1.4.6 Survival

Body weight was similar in the September and October batches. Mean survival time was markedly different between the two groups [227 ± 73 vs. 135 ± 45, Sept vs. Oct, respectively]. None of the animals survived more than 4h in the October batch [Table 6-6; Figure 6-18].
<table>
<thead>
<tr>
<th>Variable</th>
<th>September 30/20 [PIP/RR]</th>
<th>October 30/20 [PIP/RR]</th>
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<tr>
<td>Number of animals</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Animal Weight [g]</td>
<td>358 ± 17</td>
<td>358 ± 13</td>
</tr>
<tr>
<td>Animal survival at 6 h [%]</td>
<td>1/7 [14 %]</td>
<td>0/5[0%]</td>
</tr>
<tr>
<td>Duration of survival [minutes]</td>
<td>227 ± 73</td>
<td>135 ± 45</td>
</tr>
<tr>
<td>Mean Arterial Pressure [mmHg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>136 ± 17</td>
<td>155± 27</td>
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<tr>
<td>1 Hours</td>
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<td>Arterial O₂ tension [kPa]</td>
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</tr>
<tr>
<td>Baseline</td>
<td>19.0 ± 0.9</td>
<td>18.8 ± 0.9</td>
</tr>
<tr>
<td>1 Hours</td>
<td>17.4 ± 0.9</td>
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</tr>
<tr>
<td>2 Hours</td>
<td>14.3 ± 4.1</td>
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<td>3 Hours</td>
<td>11.2 ± 5.2</td>
<td>14.1</td>
</tr>
<tr>
<td>4 Hours</td>
<td>10.1 ± 5.5</td>
<td>-</td>
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<td>5 Hours</td>
<td>15.7</td>
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<td>6 Hours</td>
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<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>4.6 ± 0.4</td>
<td>4.8 ± 0.7</td>
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<td>6 Hours</td>
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<td>Arterial pH</td>
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</tr>
<tr>
<td>Baseline</td>
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<td>7.40 ± 0.06</td>
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<td>7.45 ± 0.06</td>
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<td>3 Hours</td>
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</tr>
<tr>
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<td>7.16 ± 0.19</td>
<td>-</td>
</tr>
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<td>5 Hours</td>
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<td>-</td>
</tr>
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<td>6 Hours</td>
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<td>Serum Bicarbonate [mmol/l]</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.5 ± 0.6</td>
<td>22.6 ± 1.4</td>
</tr>
<tr>
<td>Final</td>
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<td>18.8 ± 1.9</td>
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<td>Base Excess [mmo/l]</td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>-1.8 ± 1.0</td>
<td>-2.6 ± 1.4</td>
</tr>
<tr>
<td>Final</td>
<td>-13 ± 8.8</td>
<td>-8.2 ± 2.3</td>
</tr>
<tr>
<td>Serum Lactate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.0 ± 0.5</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Final</td>
<td>8.7 ± 8.2</td>
<td>7.8 ± 2.4</td>
</tr>
<tr>
<td>Static Lung Compliance [ml/mmHg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.96 ± 0.14</td>
<td>1.01 ± 0.17</td>
</tr>
<tr>
<td>Final</td>
<td>0.58 ± 0.12</td>
<td>0.67 ± 0.19</td>
</tr>
<tr>
<td>Compliance change [%]</td>
<td>39 ± 11</td>
<td>34 ± 17</td>
</tr>
</tbody>
</table>
Table 6-6 Demonstration of variability between two batches of animals [September vs. October] ventilated with the same injurious ventilation. Peak inspiratory pressure [PIP]: 30 cmH₂O, respiratory rate [RR]: 20/minute.

![Graph showing survival over time in animals from September vs. October batch with the same ventilator setting. PIP: 30 cmH₂O; RR: 20/min.](image)

Figure 6-18 Survival over time in animals from September vs. October batch with the same ventilator setting [PIP: 30 cmH₂O; RR: 20/min].

6.4.1.4.7 Arterial pCO₂ and acid base

Arterial CO₂ tension was lower and pH was higher in the October group. In spite of the higher mortality rate in the October batch, serum bicarbonate, base excess decreased less than in the September batch [Table 6-6].

6.4.1.4.8 Lung injury

Arterial pO₂ tension decreased more rapidly in the October batch and this was associated with worst oxygenation on FiO₂: 1.0 at the end of the experiment [37.7 ± 25 vs. 21.1 ± 23.5 kPa, Sept vs. Oct-, respectively] [Table 6-6; Figure 6-19]. Contrary, percentage reduction of static compliance from baseline was worst in the September batch than the October batch [39% vs. 34%, Sept vs. Oct] [Table 6-6; Figure 6-20].
Figure 6-19 Final arterial O$_2$ tension [paO$_2$] in animals receiving FiO$_2$:1.0. September vs. October batch [37.7 ± 25.5 vs. 21.1 ± 23.5 kPa] with the same ventilator setting [PIP: 30 cmH$_2$O; RR: 20/min].

Figure 6-20 Evolution of lung static compliance over time in animals from September vs. October batch with the same ventilator setting [PIP: 30 cmH$_2$O; RR: 20/min].
6.4.1.5 Series V. Evaluation of the dynamics of ventilation induced lung injury over time.

Animals subjected to PIP: 30cmH\textsubscript{2}O and RR: 20/min for 6h were included in this analysis \[n=13\]. We looked at how survival and physiological injury parameters changed over time during injurious mechanical ventilation \[Table 6-7\].

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>2 Hours</th>
<th>4 Hours</th>
<th>6 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>13/13 [100%]</td>
<td>11/13 [85%]</td>
<td>5/13 [38%]</td>
<td>2/13 [15 %]</td>
</tr>
<tr>
<td>MAP [mmHg]</td>
<td>146 ± 20</td>
<td>108 ± 19</td>
<td>99 ± 19</td>
<td>100 ± 21</td>
</tr>
<tr>
<td>(\text{paO}_2) [kPa]</td>
<td>18.8 ± 0.87</td>
<td>13.9 ± 4.2</td>
<td>11.2 ± 4.7</td>
<td>10.7 ± 4.3</td>
</tr>
<tr>
<td>(\text{paCO}_2) [kPa]</td>
<td>4.5 ± 0.5</td>
<td>3.9 ± 0.9</td>
<td>4.4 ± 1.2</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>pH</td>
<td>7.42 ± 0.04</td>
<td>7.36 ± 0.11</td>
<td>7.27 ± 0.18</td>
<td>7.26 ± 0.18</td>
</tr>
<tr>
<td>(\text{HCO}_3) [mmol/l]</td>
<td>23.0 ± 0.90</td>
<td>18.8 ± 3.1</td>
<td>17.0 ± 4.4</td>
<td>16.4 ± 5.2</td>
</tr>
<tr>
<td>BE [mmol/l]</td>
<td>-2.3 ± 1.2</td>
<td>-7.6 ± 4.0</td>
<td>-9.6 ± 5.6</td>
<td>-10.2 ± 6.5</td>
</tr>
<tr>
<td>Serum Lactate [mmol/l]</td>
<td>2.1 ± 0.4</td>
<td>5.7 ± 2.8</td>
<td>7.5 ± 4.7</td>
<td>7.3 ± 4.9</td>
</tr>
<tr>
<td>Static compliance [ml/mmHg]</td>
<td>1.0 ± 0.14</td>
<td>0.67 ± 0.17</td>
<td>0.65 ± 0.15</td>
<td>0.62 ± 0.16</td>
</tr>
</tbody>
</table>

\textbf{Table 6-7} Evaluation of ventilation induced lung injury over time in rats ventilated with peak inspiratory pressure [PIP]: 30 cmH\textsubscript{2}O and respiratory rate [RR]: 20/minute.

6.4.1.5.1 Survival

Survival decreased gradually to 85, 38, 15\% after 2, 4, and 6h ventilation \[Figure 6-21\]. Mean survival time was 200 ± 96 min.
Figure 6-21 Animal survival over time at baseline, 2, 4 and 6 hours during injurious ventilation with PIP: 30 cmH$_2$O and RR: 20/min.

6.4.1.5.2 Arterial pCO2 and acid base

Arterial CO$_2$ tension did not change markedly from baseline, however, arterial pH decreased from 7.42 ± 0.04 to 7.26 ± 0.18 over 6h [Figure 6-22; Figure 6-23]. This was associated with significant decrease in serum bicarbonate, base excess levels, and a gradual increase in serum lactate levels [Table 6-7].

Figure 6-22 Arterial CO$_2$ tension [paCO$_2$] over time at baseline, 2, 4 and 6 hours after injurious ventilation with PIP: 30 cmH$_2$O and RR: 20/min.
Arterial pH over time at baseline, 2, 4 and 6 hours during injurious ventilation with PIP: 30 cmH2O and RR: 20/min.

6.4.1.5.3 Lung injury

Arterial pO2 tension decreased gradually from 18.8 ± 0.87 to 10.7 ± 4.3 kPa during the experiment and this was associated with gradual decrease in static compliance [38%] compared to baseline values [Figure 6-24; Figure 6-25].

Arterial O2 tension [pO2] over time at baseline, 2, 4 and 6 hours during injurious ventilation with PIP: 30 cmH2O and RR: 20/min.
6.4.1.6 Series VI. Comparison of injury severity resulting from moderate and severe VILI ventilation protocols.

Respiratory settings of PIP: 30 cmH$_2$O, RR: 15/min were defined as moderate VILI [30/15] and PIP: 30cmH$_2$O, RR: 18/min were defined as severe VILI [30/18] [Table 6-8]. In the final models, we took into account the major conclusions derived from the pilot experiments. Both animal groups were younger animals with BW <400 g, we used animals strictly from the same batch. We applied PIP of 30 cmH$_2$O, since this resulted in significant but gradual injury over 6 hours. We choose to carry out our experiments over 4h based on the results of survival patterns from the pilot data. A respiratory rate of 15-20/min resulted in normocapnia, therefore those settings were suitable to apply in the control group for the final experimental series.
<table>
<thead>
<tr>
<th>Variable</th>
<th>30/15</th>
<th>30/18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Animal Weight [g]</td>
<td>373 ± 27</td>
<td>399 ± 27</td>
</tr>
<tr>
<td>Mean survival time [min]</td>
<td>240</td>
<td>166 ± 69</td>
</tr>
<tr>
<td>Survival at 4 h [%]</td>
<td>6/6 [100%]</td>
<td>3/7 [43%]</td>
</tr>
<tr>
<td>Mean arterial pressure [mmHg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>154 ± 15</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>1 Hours</td>
<td>119 ± 19</td>
<td>119 ± 24</td>
</tr>
<tr>
<td>2 Hours</td>
<td>137 ± 20</td>
<td>109 ± 33</td>
</tr>
<tr>
<td>3 Hours</td>
<td>129 ± 22</td>
<td>105 ± 31</td>
</tr>
<tr>
<td>4 Hour</td>
<td>112 ± 21</td>
<td>94 ± 35</td>
</tr>
<tr>
<td>Arterial O₂ tension [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>18.3 ± 0.8</td>
<td>18.3 ± 0.8</td>
</tr>
<tr>
<td>1 Hours</td>
<td>16.5 ± 1.1</td>
<td>16.8 ± 0.8</td>
</tr>
<tr>
<td>2 Hours</td>
<td>14.9 ± 1.3</td>
<td>13.8 ± 4.8</td>
</tr>
<tr>
<td>3 Hours</td>
<td>13.2 ± 1.5</td>
<td>12.9 ± 4.3</td>
</tr>
<tr>
<td>4 Hours</td>
<td>11.1 ± 3.9</td>
<td>12.3 ± 4.7</td>
</tr>
<tr>
<td>Arterial O₂ tension on FiO₂:1.0 [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>41.3 ± 23.6</td>
<td>23.7 ± 23.7</td>
</tr>
<tr>
<td>Arterial CO₂ tension [kPa]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.8 ± 0.3</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>1 Hours</td>
<td>4.7 ± 0.8</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>2 Hours</td>
<td>5.4 ± 0.4</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>3 Hours</td>
<td>5.7 ± 0.9</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>4 Hours</td>
<td>6.1 ± 0.7</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.41 ± 0.03</td>
<td>7.40 ± 0.04</td>
</tr>
<tr>
<td>1 Hours</td>
<td>7.39 ± 0.05</td>
<td>7.45 ± 0.02</td>
</tr>
<tr>
<td>2 Hours</td>
<td>7.33 ± 0.06</td>
<td>7.35 ± 0.11</td>
</tr>
<tr>
<td>3 Hours</td>
<td>7.30 ± 0.08</td>
<td>7.34 ± 0.12</td>
</tr>
<tr>
<td>4 Hours</td>
<td>7.27 ± 0.11</td>
<td>7.32 ± 0.13</td>
</tr>
<tr>
<td>Serum Bicarbonate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.4 ± 0.9</td>
<td>23.3 ± 1.3</td>
</tr>
<tr>
<td>Final</td>
<td>19.7 ± 4.5</td>
<td>16.7 ± 4.4</td>
</tr>
<tr>
<td>Base Excess [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-1.6 ± 0.9</td>
<td>-1.7 ± 1.4</td>
</tr>
<tr>
<td>Final</td>
<td>-5.0 ± 5.1</td>
<td>-9.8 ± 5.2</td>
</tr>
<tr>
<td>Serum lactate [mmol/l]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.4 ± 0.4</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Final</td>
<td>4.2 ± 3.2</td>
<td>8.7 ± 5.0</td>
</tr>
<tr>
<td>Static Compliance [ml/mmHg]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.95 ± 0.04</td>
<td>1.15 ± 0.18</td>
</tr>
<tr>
<td>Final</td>
<td>0.69 ± 0.11</td>
<td>0.73 ± 0.22</td>
</tr>
<tr>
<td>Compliance change [%]</td>
<td><strong>27 ± 12</strong></td>
<td><strong>35 ± 19</strong></td>
</tr>
<tr>
<td>BAL total cell count [cell/ml]</td>
<td>53167 ± 21711</td>
<td>30286 ± 10610</td>
</tr>
<tr>
<td>Percentage of neutrophil in BAL [%]</td>
<td>35 ± 13</td>
<td>55 ± 24</td>
</tr>
<tr>
<td>BAL neutrophil count [cell/ml]</td>
<td>16817 ± 5696</td>
<td>14490 ± 3514</td>
</tr>
</tbody>
</table>

**Table 6-8** Comparison of final VILI models. *Moderate VILI* [PIP: 30 cmH₂O, RR: 15/minute], *Severe VILI* [PIP: 30 cmH₂O, RR: 18/minute].
6.4.1.6.1 Survival

All animals survived the 4h experimental period in the moderate VILI group. In contrast with this, in the severe VILI group, animals started to die after 1h injurious ventilation, resulting in a survival rate 71, 43, 43 % at 2, 3, and 4 hours respectively [Figure 6-26]. Mean survival time was 166 ± 69 min in the severe VILI group [Table 6-8; Figure 6-27]. There were no differences between the groups at baseline with regard to arterial O$_2$, CO$_2$ tensions, arterial pH, serum lactate and bicarbonate. Static compliance was slightly higher at baseline in the severe VILI than in the moderate VILI group [Table 6-8].

![Figure 6-26](image.png)

**Figure 6-26** Animal survival over time ventilated with PIP: 30 cmH$_2$O and RR: 15/min versus PIP: 30 cmH$_2$O /RR: 18/min.
Arterial CO$_2$ tension increased gradually in both groups over time and was higher at each time point in the moderate VILI series [Figure 6-28]. However, arterial CO$_2$ tension was in the normal range in both experimental groups. In parallel with this, arterial pH was lower in the moderate VILI group throughout the experiment [Figure 6-29]. Serum bicarbonate and base excess decreased more in the severe VILI group and this was associated with a more marked increase in serum lactate levels at the end of the experiment [4.2 ± 3.2 vs. 8.7 ± 5.0 mmol/l, moderate VILI vs. severe VILI] [Table 6-8; Figure 6-30].
Figure 6-28 Arterial CO₂ tension [paCO₂] over time in rats ventilated with PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min.

Figure 6-29 Arterial pH over time in rats ventilated with PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min.
Figure 6-30 Serum lactate levels over time in rats ventilated with PIP: 30 cmH\textsubscript{2}O and RR: 15/min vs. PIP: 30 cmH\textsubscript{2}O and RR: 18/min.

### 6.4.1.6.3 Lung injury

Initially, arterial pO\textsubscript{2} decreased more rapidly in the severe VILI group, however at 3h and 4h paO\textsubscript{2} was slightly higher in the severe VILI group than in the moderate VILI group [Figure 6-31]. Final paO\textsubscript{2} on FiO\textsubscript{2}: 1.0 was higher in the moderate VILI group than in the severe VILI group [41.3 ± 23.6 vs. 23.7 ± 23.7 kPa] [Figure 6-32]. Static compliance decreased in both group gradually over time, however percentage decrease was worst in the severe VILI than in the moderate VILI group, 35 % vs. 27 %, respectively [Table 6-8; Figure 6-33]. Lung wet/dry ratio was higher in the severe VILI group representing more oedema formation in comparison to the moderate VILI [Figure 6-34]. BAL neutrophil count was similar between the two groups, however the percentage of neutrophils were higher in the severe VILI group [55% vs. 35 %, severe VILI vs. moderate VILI] [Table 6-8].
Arterial O₂ tension [paO₂] over time in rats with PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min.

Arterial O₂ tension [paO₂] in rats receiving FiO₂: 1.0 after 4 hours injurious ventilation. PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min.
Figure 6-33 Evolution of static compliance over time in rats ventilated with PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min.

Figure 6-34 Lung wet/dry ratio after 4 hours injurious ventilation. PIP: 30 cmH₂O and RR: 15/min vs. PIP: 30 cmH₂O and RR: 18/min [5.8 ± 1.8 vs. 7.4 ± 1.4].
6.5 DISCUSSION

The main advantage of VILI model is its clinical relevance. Lessons from this model resulted in the realization of the ARMA trial and ultimately, improved survival in ARDS patient [5]. The main disadvantage of the VILI model is its complexity and this is closely linked to the pathomechanism of the injury and the species specific differences in anatomical structures [see Chapter 3, Section 3.3].

In order to investigate how HCA affects VILI, the model should incorporate and express the pathophysiological changes related to both mechanical and inflammatory injury. The ideal ventilation setting and time to produce such changes without significant mortality, however, is unclear. In our pilot work we aimed to explore the possible factors which determine the evolution of VILI and to develop a gradual lung injury model in Sprague-Dawley rats over 4-6h period of time.

6.5.1 IMPACT OF AGE OF THE ANIMALS ON THE DYNAMICS OF VILI

Younger animals seemed to tolerate injurious ventilation more than older animals. Mean survival time in older animals was shorter than younger animals, and this was associated with lower MAP, higher lactate, lower serum bicarbonate and base excess levels after 6h mechanical ventilation. Arterial oxygenation decreased more markedly in older animals, accompanied by higher CO₂ levels and lower pH. Lung mechanics was worst in older animals compared to younger animals.

6.5.1.1 Preclinical studies

There is little information regarding the impact of age on VILI models. Most of the experimental studies compared differences in susceptibility between newborns and adult animals [271, 272]. The general concept, although not unanimously [317], states that newborn animals exposed to high Vₜ ventilation are more resistant and react with less inflammation than adult animals. Most recently, Nin et al demonstrated that the adverse effects of high Vₜ [35 ml/kg] combined with high RR [70/min] ventilation were more severe in old than in young animals evidenced by higher histological lung injury scores, higher serum IL-6 levels, and worst arterial oxygen tension. Mortality rate was 66% and was due to severe circulatory shock in old animals. Importantly, older rats had higher P_peak and P_mean both at low and high Vₜ ventilation than younger rats. Similar
observations have been reported by Malus et al in an ex vivo rat model of VILI. Excised lungs from rats weighing 200 and 400g ventilated with identical $V_t$ [30 ml/kg] resulted in higher $P_{\text{plateau}}$ and TNF-α levels in the lungs excised from larger [older] animals [318].

6.5.1.2 Age related changes in lung and immune physiology

Lung mechanics changes with age. These include: decreased lung and chest compliance and reduced strength of the respiratory muscles [319]. Structural changes in the spatial arrangement and cross-linking of elastic fibres [320] and homogenous enlargement of airway spaces result in decreased surface area of airspace wall per unit of lung volume[321, 322]. These alterations are similar to those seen in emphysema, featured with leftward and upward shift of the pressure-volume curve, increased residual volume and FRC with relatively maintained total lung capacity [319; 321]. Therefore, older subjects breathe at higher lung volumes and may be more susceptible to over distension in response to high $V_t$ ventilation than young subjects. The loss of supporting tissues around the airways increases closing volume and may equal FRC. Premature closure in the small airways even at normal $V_t$ leads to low V/Q ratio, reduced $pao_2$, increased $AaO_2$ difference and reduced diffusion capacity [319].

Older age also associated with impaired immune response to noxious stimuli [276, 323]. Gradual loss of naive CD4$^+$ and CD8$^+$ cells during thymus involution leads to impaired T cell function with abnormal shift towards increased levels of IL-4, IL-6, IL-10 and IL-15 [324]. This is also accompanied with substantially decreased B cell population and function [276, 323, 367]. As a result of these, cell mediated immune defences against previously recognized pathogens may remain intact, however, effective immune responses to new antigens is decreased [276, 367].

Innate immunity represents the first line of host defence against invading pathogens. Although absolute number of neutrophils in the blood, neutrophil precursors in the bone marrow , chemotaxis and neutrophil adhesion are well-preserved in the elderly [367, 370], neutrophil function, such as phagocytosis of opsonized bacteria [E. Coli and S. aureus], Fc-receptor mediated superoxide generation and phagocytosis are significantly impaired in the elderly population [368]. Similarly, macrophage mediated antigen presentation have been shown to be altered due to reduced levels of MHC-II
molecules, which may adversely influence CD4+ dependent T cell response during aging [367, 369]. Reduced respiratory burst activity of macrophages is also an important contributor to age related immune dysfunction. Most importantly, it has been shown that circulating levels of pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α, are chronically elevated in elderly people, resulting in a subclinical and persistent inflammatory status [367]. This may result in more prolonged pro-inflammatory response in elderly than younger patients and higher cytokine response to injury [276].

Taken together, age related changes in lung function coupled with dysfunctional immune response to noxious stimuli may explain partially the differences in sensitivity to mechanical stretch in the different age groups in our study.

### 6.5.1.3 Limitations

Although our data supports the observations of Nin et al [273], there are number of limitations to our analysis: (1) our experiments were not prospective; (2) we choose to analyse experiments from the 25/20 ventilator setting [very moderate VILI], because these were the initial piloting studies and included animals with larger BW and older age range than later experiments; (3) we had unequal number of animals in the two groups and experiments included rats from different batches; (4) the difference in age between younger and older animals was not as marked in our data. Average BW was 358±19 g in the younger animal group and 447±24 g in the older animal group, representing an average of 3-4 weeks of age difference between the two groups. In contrast with this, Nin et al compared animals with larger age difference [3-4 months vs. 22-24 months old] and employed more injurious ventilation strategy [tidal volume: 35ml/kg; RR: 70 /min][273]. Nevertheless, data from human and animal studies strongly supports that age plays a major role determining the magnitude of VILI, and indicate that it is important to standardise rat age when conducting studies in ventilation induced lung injury model.

### 6.5.2 EFFECT OF PEAK INSPIRATORY PRESSURE ON THE SEVERITY OF VENTILATION INDUCED LUNG INJURY.

Increasing PIP from 25 to 27.5 cmH₂O caused mild hypoxaemia over time, while 30 cmH₂O resulted in severe hypoxaemia, circulatory shock and earlier mortality in rats.
Static lung compliance decreased moderately, and nearly equal manner in the groups ventilated with 25 and 27.5cmH₂O PIP. Static compliance reduction was more pronounced in animals ventilated with 30cmH₂O of PIP. All animals ventilated with 25 cmH₂O and 27.5cmH₂O of PIP survived the 6h ventilation protocol while animals receiving 30cmH₂O of PIP started to die after 3h mechanical ventilation. Our data indicates that the threshold pressure, at which injury starts to accelerate in rats, is over 27.5cmH₂O and the timeframe to cause significant lung injury is between 2-4 hours.

The importance of the threshold pressure lies in the fact that the injury dynamics is exponential rather than linear in VILI [50, 119]. This means that obvious, measurable pathophysiological changes become apparent suddenly once the distending pressure reaches a certain value. Above this pressure there is more chance to detect consistently the cardinal pathological changes of ALI. This has been repeatedly demonstrated in the literature and can be summarised as follows: (1) In in vivo rat models of VILI, injury starts to accelerate at around 30cmH₂O of PIP and usually associated with obvious macroscopic and microscopic changes after 1-3h ventilation [51, 113]. PIP reaching 37-40cmH₂O cause very rapid injury within 30 minutes and early death [51, 115]. Importantly, lower PIP (~22cmH₂O) is not associated with significant lung physiological alterations even after 7h mechanical ventilation, however, it still causes increased BAL neutrophil recruitment indicating that inflammatory response to mechanical ventilation is present even at lower intensity ventilation [115]; (2) In ex vivo dog [119] and rabbit [316] lung preparations threshold pressure were around 27-30 cmH₂O. Lung microvascular permeability increased exponentially above these airway pressures; (3) In in vivo lamb model, increasing Vt were associated with sudden increase in lymph/plasma protein ratio and decrease in albumin-globulin ratio in the lymph and plasma at Vt 45 ml/kg, representing 43cmH₂O of PIP[82].

These studies and ours highlights that the existence of a pressure “threshold” for injury, rather than a linear worsening of injury with increasing PIP, makes difficult to alter lung injury severity by changing the PIP.
6.5.3 EFFECT OF DIFFERING RESPIRATORY RATES ON VENTILATION INDUCED LUNG INJURY.

Lower RR was associated with less severe VILI when same peak inspiratory pressure was applied. Animals ventilated with RR: 15/min had more prolonged survival and were less hypotensive than animals ventilated with RR: 20/min. Arterial oxygenation varied between the two groups, and after 2h ventilation PaO₂ tension was lower in the 30/15 group. Arterial CO₂ tension was marginally higher and pH was lower in the 30/15 group, however, CO₂ levels remained in the normal range in both groups. Acid base and lactate levels were not different between the two groups. Static compliance decreased more in the 30/20 than in the 30/15 [38 % vs. 29 %] group. BAL neutrophil counts were similar between the two groups.

Most of the research interest has been focused on the role of overdistension and atelectrauma in VILI. The contribution of dynamic components of mechanical ventilation, such as RR, inspiratory flow rate or inspiratory flow pattern on VILI is not clear. This may be explained by the fact, that it is difficult to investigate in isolation these factors due to their close relationship to inspiratory time, inspiratory to expiratory ratio, and Vt. In addition, studies addressing the effect of RR on VILI include different ventilation strategies, such as volume vs. pressure control ventilation, constant vs. decelerating inspiratory flow rate, presence or absence of PEEP. In the present experimental design, we applied pressure control ventilation with pressure cycle and constant inspiratory flow ventilation. This meant that during inspiration, flow rate remained constant and inspiration changed to expiration when PIP reached the target pressure, in this case 30 cmH₂O. RR determined the total time of respiratory cycle, however, the inspiratory time to reach the set PIP was also dependent on the actual lung compliance and the airway resistance. Therefore, both Vt and exposure time to injurious stretch varied over time.

6.5.3.1 Preclinical studies

The first report that specifically looked at the effect of high RR on lung function was carried out on spontaneously breathing animals [325]. Following repeated infusion of sodium salicylate into the cistern magna, adult sheep developed hyperventilation leading to hypoxaemia, decreased static compliance, abnormal chest radiograph, and
gross macroscopic changes in the lungs. Increased minute ventilation was related to increased RR rather than increased $V_t$. *Ex vivo* experiments in dog [326], rabbit [327] and rat [328] lungs have also confirmed that the severity of VILI can be modified by altering RR. *Rich et al* have demonstrated in an *in vivo* rat model that high $V_t$ [40 ml/kg] with higher RR [40/min] resulted in worst lung mechanics and higher BAL cytokine levels than lower RR [20/min]. In contrast, normal $V_t$ [7ml/kg] ventilation did not cause significant lung injury even when the RR was increased to 40/min [329]. A most recent, *in vivo* study has shown that increasing RR [80, 120, 160/min] at clinically more relevant tidal volumes [8, 10, 12 ml/kg] did not influence respiratory mechanics. However, moderate changes in histology, BAL IL-6 levels and activation of intracellular mechanotransduction pathways [AKT and ERK 1/2] became activated when RR was higher [330]. This indicates that inflammatory response to mechanical stretch is ongoing even in the absence of gross changes in lung mechanics or oxygenation and at least partially depends on RR.

Overall, these studies suggest that RR can independently influence injury severity and survival in animals. Importantly, the measured effect depends on the magnitude of the applied pressure or volume [328, 329]. RR does change VILI dramatically when the applied pressure or volume is high, while alterations in RR at lower pressure/volume ventilation are more tolerable and do not result in such severe lung injury.

With regard to our model we can conclude: (1) modifying RR to change injury severity and survival is most effective when the applied pressure/volume is high [i.e. above threshold pressure]; (2) it seems that the most important factors determining injury dynamics are the combination of RR/PIP [in the context of pressure control ventilation] and exposure time. These components would express the intensity of mechanical ventilation - also the intensity of stretch and strain on the lung tissue - and determine the outcome of the VILI model; (3) based on these observation we used RR to modify injury severity in our final VILI models. Setting PIP above the threshold pressure [30 cmH$_2$O], a RR 15/min resulted in *moderate*, while a RR: 18/min resulted in *severe* VILI.
6.5.4 IMPACT OF DIFFERING ANIMAL BATCHES ON VENTILATION INDUCED LUNG INJURY.

Inter-batch variability was examined comparing batches retrospectively in two separate experimental settings [30/15 and 30/20]. The difference between the March vs. April batches in terms of survival, oxygenation, CO\(_2\) elimination and acid base did not seem to be significant, while static compliance decreased more in the April batch. In contrast, September vs. October batches responded very differently to injurious ventilation. Animals from the September batch tolerated VILI much better and had better survival and oxygenation.

Sprague-Dawley rats are one of the most frequently used outbred animal stocks in experimental research. The genetic variability in these animals depends on the previous history of the colony. The magnitude of the genetic variation is generally unknown unless it is specifically tested [331]. This means that an animal assigned to a control group will be genetically different from one assigned to a treatment group. Any differences in the response to the intervention may be due to the effect of the treatment or to the difference of their genetic makeup. It is important to know that the characteristics of the colony can change over time as a result of mixing with other outbred stocks, inbreeding, natural selection, and new mutations [332]. Genotypic changes, however, may only manifest after several generations [333]. Because of the constant changing of genetic makeup in outbred animals, it is understandable that batch to batch variability can affect experimental outcome.

Our analysis had several limitations. We did not examine the effect of differing batches on VILI per se, and the data was analysed retrospectively. Also, because we noticed that some batches are more susceptible than others, the number of animals included in the October batch group was very low simply because we stopped performing experiments on that particular batch. Despite of the limitations, batch-dependent response to VILI is clear, and it seems reasonable to reduce variability to experimental conditions by performing experiments on animals from the same batch.
6.5.5 Evaluation of the Dynamics of Ventilation Induced Lung Injury Over Time.

In this analysis we wished to look at the evolution of VILI over 6 hours in 13 animals. We included experiments with a respiratory setting of PIP: 30 cmH\textsubscript{2}O and RR: 20/min. Based on these observations we concluded that VILI resulted in progressive alterations in the most important injury indices: (1) survival, MAP, serum lactate levels, acid base balance; (2) arterial oxygenation and static compliance. We also needed to determine how PaCO\textsubscript{2} changes over time with this setting in order to generate a model where the control group would express normocapnia in subsequent experiments. PaCO\textsubscript{2} levels varied over time but remained in the lower range of normal values. Overall, we have decided to use slightly different settings for our final experiments for the following reasons: (1) our aim was to generate a 4h VILI model with acceptable survival. RR: 18 and 15/min seemed to result in better survival at 4h than the RR: 20/min; (2) Lower RR resulted in slightly higher but still normal paCO\textsubscript{2} levels, which were more suitable for control groups in later experiments; (3) PIP: 30cmH\textsubscript{2}O ventilation was repeatedly associated with significant lung injuries indices. Based on these, we defined two injury models. In the moderate VILI experiments animals were ventilated with PIP: 30 cmH\textsubscript{2}O and RR: 15/min and in the severe VILI series with PIP: 30 cmH\textsubscript{2}O and RR: 18/min.

6.5.6 Moderate Versus Severe VILI

We have determined two injury severities with identical PIP [30cmH\textsubscript{2}O] but distinct respiratory rates. In the severe VILI model RR was 18/min, while in the moderate VILI model 15/min. At first glance it seems that the difference between RR is too little to generate such difference in injury severity, however, comparing the two respiratory settings, we could demonstrate that RR: 15/min was associated with much less injury evidenced by better oxygenation, compliance, lactate levels, acid base and better survival than RR: 18/min.

Previous studies in the literature and the analysis of our pilot studies suggest that RR can independently influence injury severity and survival in animals [325-330]. The contribution of RR in “fine tuning” of injury severity in an animal model, however, can be most efficiently exploited when the applied PIP is over the threshold pressure, in our case 27.5 cmH\textsubscript{2}O. Also, exposure time, 4h of VILI, seemed to be sufficient to
demonstrate the difference between the two injurious ventilation settings. Furthermore, it is also important to see if the model represents the clinical scenario or not. Mechanical ventilation employing PIP: 30 cmH\(_2\)O and RR: 15 or 18/min are not uncommon in clinical practice, and P\(_\text{plat}\) or P\(_\text{peak}\) less than 35 cmH\(_2\)O have been used in lung protective clinical studies in ALI/ARDS [5, 67, 120-122].

In summary, we have developed an in vivo rodent model that was suitable to measure the effect of HCA on VILI in two distinct severities. We took into consideration controllable factors, such as age and batch, and also determined the optimal injury time and severity to reliably demonstrate the main characteristics of ALI.

### 6.6 CONCLUSIONS

The following observations have been considered during model development:

1. **Choice of animal species.** In vivo rat model of VILI is reproducible, suitable and feasible to investigate the effect of injurious mechanical ventilation over 4h.

2. **The age of the animal.** Age influences injury dynamics and can introduce variability.

3. **Threshold pressure.** Lung injury starts to accelerate above PIP: 27.5cmH\(_2\)O, and results in obvious changes in respiratory mechanics, gas exchange and inflammation.

4. **Respiratory rate.** RR influences injury severity mainly above the threshold pressure. RR: 15/min resulted in moderate, while RR: 18/min in severe VILI when PIP: 30cmH\(_2\)O was applied.

5. **Animal batch.** Inter batch variability can influence experimental outcome therefore experiments should preferably be carried out using animals from the same batch.

6. **Survival rate and evolution of VILI.** Mechanical ventilation with PIP:30 cmH\(_2\)O leads to increasing mortality between 2-3h, however, 40-50% of the animals are still alive at 4h. Four hour experimental period is suitable to achieve VILI with altered physiological and inflammatory parameters.
CHAPTER 7
CHAPTER 7: HYPERCAPNIC ACIDOSIS ATTENUATES MODERATE AND SEVERE VENTILATION INDUCED LUNG INJURY VIA NF-κB PATHWAY

7.1 ABSTRACT

Background: Hypercapnic acidosis protects against lung injury in several contexts, including ischaemia-reperfusion, sepsis, and ventilation induced lung injury. However, HCA may delay wound repair and impair the host immune response. Both the beneficial and deleterious effects of HCA may be mediated via inhibition of the NF-κB pathway. NF-κB is ubiquitously expressed transcription factor initiating inflammatory gene expression in response to various stimuli, including high mechanical stretch. HCA has been shown to inhibit the degradation of IkB-α, the main regulatory step of the NF-κB pathway, in various in vitro injury models, including sepsis and wound repair. We hypothesised, that the protective, anti-inflammatory effect of HCA in in vivo rodent model of VILI is mediated by NF-κB inhibition.

Methods: Adult, male Sprague-Dawley rats were utilized. In separate experimental series, the potential for HCA to attenuate VILI of different severities was determined. In the severe VILI series [n=14], animals underwent ventilation with peak inspiratory pressure [PIP]: 30 cmH2O, PEEP: 0 cmH2O, and respiratory rate [RR]: 18 breaths/min for 4 hours. In the moderate VILI series [n=12], animals underwent ventilation with PIP: 30 cmH2O, PEEP: 0 cmH2O at a RR: 15 breaths/min. Survival, haemodynamic profile, severity of lung injury and indices of activation of the NF-κB pathway were assessed.

Results: HCA attenuated both severe and moderate VILI. However, the magnitude of the protective effect on physiological and inflammatory markers was more pronounced in animals receiving the more aggressive ventilation strategy. In the severe VILI series, HCA significantly prolonged survival, resulted in higher mean arterial blood pressure and lower lactate levels compared to normocapnia. HCA significantly improved arterial oxygenation, alveolar permeability, and indices of inflammation, such as bronchoalveolar lavage [BAL] IL-6, CINC-1, TNF-α, and BAL neutrophil infiltration. Histological injury was also attenuated in the HCA group. The protective effects of HCA were seen in the context of reduced activation of the NF-κB pathway. In addition, HCA prevented the degradation of cytoplasmic IkB-α in the moderate VILI.
series, maintaining cytoplasmic IκB-α concentrations at levels comparable to that seen in control non-ventilated animals. In conjunction with this, HCA reduced p65 nuclear transport compared to normocapnia.

**Conclusion:** Our findings demonstrate that HCA inhibits mechanical stretch induced lung injury by an NF-κB dependent mechanism.
7.2 INTRODUCTION

One of the most important findings in ALI/ARDS research was the demonstration that mechanical ventilation – while a necessary and life-saving intervention - can do harm and worsen ALI. The consequent scientific efforts all aimed to minimize mechanical stretch related injury to the lungs and resulted in the introduction of lung protective ventilation strategies, application of PEEP and acceptance of permissive hypercapnia in the management of ALI/ARDS. The protective effect of the above mentioned strategies have been supported by solid preclinical evidence [50, 51, 71, 73] and some clinical data [1, 5, 48, 67, 69]. However, translation of stretch induced signals into biological responses and the contribution of protective strategies on these signal transduction channels remains to be elucidated. In our study we wished to investigate how HCA modulates the NF-κB pathway and how this is translated into protection against mechanical stretch induced ALI. These questions have stemmed from observations from previous studies: (1) HCA has been proved to be protective in various ex vivo and in vivo ALI models [42, 126-128, 130, 131, 139, 187], including VILI [88, 89, 125, 138]; (2) the secondary analysis of data from the ARMA trial suggested that permissive hypercapnia may have improved survival in patients randomized to high V_t ventilation [69]; (3) NF-κB pathway regulates gene expression central to lung inflammation, injury, and repair [See Chapter 1 Introduction, Section 1.5]; (4) activation of the NF-κB pathway may be the key intracellular mechanism by which excessive stretch results in cellular activation, inflammation and injury. This has been demonstrated in various in vitro, ex vivo and in vivo VILI models [91, 95-97]; (5) HCA or CO₂ attenuates sepsis induced endothelial injury, alveolar macrophage activity and lung epithelial wound repair via NF-κB inhibition in vitro [134, 136, 190].

Given the importance of the NF-κB pathway in the evolution of VILI and the possibility of hypercapnia to exert its effects through this pathway; we hypothesized that HCA would attenuate VILI at two distinct severities [moderate and severe VILI], and that HCA attenuates VILI via inhibition of the NF-κB pathway in an in vivo rodent models.
7.3 METHODS

All experiments were carried out under a licence from the Department of Health and Children, Ireland, and following approval from the Institutional Animal Care Research Ethics Committee of the National University of Ireland, Galway.

7.3.1 ANIMAL SURGERY

Adult, male Sprague Dawley rats were used in these experiments. Anesthesia was induced with intraperitoneal ketamine and xylazine. After confirming depth of anesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein and further anesthesia maintained with an intravenous Saffan infusion. A tracheostomy tube was inserted, secured and intravascular access placed in the right carotid artery. Following confirmation of depth of anesthesia using paw clamp, Cisatracurium besilate was administered intravenously to produce muscle relaxation. The animals were ventilated using a small animal ventilator with an inspired gas mixture of FiO₂: 0.3, respiratory rate of 90/min, tidal volume of 6 ml/kg, and PEEP of 2.5 cmH₂O. To minimize lung derecruitment, a recruitment maneuver consisting of a PEEP of 10 cmH₂O for 25 breaths was applied. Depth of anesthesia was assessed every 15 minutes by monitoring the cardiovascular response to paw clamp. Body temperature was maintained at 36-37.5°C using a thermostatically controlled blanket system and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures and temperature were continuously measured throughout the experimental protocol. After 20 minutes an arterial blood gas sample was drawn for blood gas measurement, and lung compliance measured in order to obtain baseline values. These measurements were repeated at hourly intervals over the course of the experimental protocol.

7.3.2 VENTILATION INDUCED LUNG INJURY PROTOCOLS

Two experimental series were carried out with two distinct mechanical ventilation protocols. In each experimental series, following induction of anesthesia, tracheostomy insertion and placement of arterial and venous access, and confirmation of baseline inclusion criteria, animals were randomized to Normocapnia
[Normocapnia; FiCO₂: 0.00] or HCA [Hypercapnic acidosis; FiCO₂: 0.05]. In the **severe VILI** series, the following settings were applied: peak inspiratory pressure [PIP] 30cm H₂O; respiratory rate [RR]: 18/min; PEEP: 0 cmH₂O for a period of 4 hours. In the **moderate VILI series**, the following settings were applied: peak inspiratory pressure [PIP]: 30 cmH₂O; respiratory rate [RR] 15/min; PEEP: 0 cmH₂O for a period of 4 hours. Exclusion and termination criteria are listed in the Materials and Methods chapter [Chapter 3] under Section 3.3.6.1.

### 7.3.3 MEASUREMENTS AND ANALYSES

A detailed description of all measurements and assays is given in the Materials and Methods chapter [Chapter 3, Section 3.5].

Physiological variables were measured as described in Section 3.5.1.

Tissue sampling and assay protocols were carried out as described in Section 3.5.2-3.5.6.

Tissue preparation and determination of NF-κB activity from total, cytoplasmic and nuclear lung tissue fraction is described under Section 3.5.7-8 and Section 3.5.9.

Histologic preparation and stereological analysis is described in Section 3.5.10-11.

### 7.3.4 STATISTICAL ANALYSIS

Distribution of data was tested for normality using the Kolmogorov-Smirnov test. Continuous variables are expressed as mean ± standard deviation [SD] for normally distributed data, and as median [interquartile range, IQR] if non-normally distributed. Response variables that were obtained at multiple time points throughout the experiments, such as paO₂, paCO₂, pH, serum lactate, static compliance, MAP were analysed by two-way analysis of variance, with group allocation as a group factor [HCA vs. Normocapnia] and time as a repeated measure. There was no evidence against normality and equal variance for these variables. Data obtained at the end of the experiments, such as wet/dry weight ration, BAL neutrophil counts, cytokine levels, total IκB-α and p65 levels were analyzed, using a Student’s t test or Mann-Whitney U test. Cytoplasmic IκB-α contents were analysed by two-way analysis of variance. Lung
histology was analyzed by two-way analysis of variance, with group as the first factor and histologic classification [ airspace, tissue space, extra-acinar tissue ] as the second factor. Post hoc tests were carried out using Mann-Whitney U test with the Bonferroni correction for multiple comparisons, as appropriate. Mortality data were analyzed using a Fisher’s exact test. A two-tailed $p < 0.05$ was considered significant.
7.3.5 RESULTS

7.3.5.1 Effects of HCA in severe VILI

Fourteen animals were entered into this study. No animals were excluded prior to randomization, and all 14 animals were randomized to receive Normocapnia [n = 7] or HCA [n = 7].

7.3.5.1.1 Baseline characteristics

There were no differences between the groups at baseline with regard to animal weight, MAP, PaO₂, PaCO₂, arterial pH, serum lactate and bicarbonate, and static compliance [Table 7-1; Figure 7-1 - 7-5].

7.3.5.1.2 Animal survival

Three animals [47%] in the Normocapnia group survived the entire protocol duration, compared to 6 animals [86%] in the HCA group [p=0.2]. HCA significantly increased the duration of animal survival compared to Normocapnia [Table 7-1].

7.3.5.1.3 Arterial CO₂ tension and Acid Base

Arterial pH and PaCO₂ were similar in the Normocapnia and HCA groups at baseline [Figure 7-1; Figure 7-2]. There was an initial rapid increase in PaCO₂ and decrease in pH in the HCA group following the induction of hypercapnia. At each hourly time point during the experiment pH was significantly lower and PaCO₂ was significantly higher in the HCA than in the Normocapnia group. Serum bicarbonate and base excess decreased significantly from baseline in both groups over the course of the protocol, but there were no between group differences [Table 7-1]. HCA significantly attenuated the increase in serum lactate over the course of the protocol, with serum lactate significantly lower at 120, 180 and 240 minutes compared to Normocapnia [Figure 7-3].

7.3.5.1.4 Hemodynamic profiles

Mean arterial pressure decreased significantly in both groups over time. HCA attenuated the decrease in MAP compared to Normocapnia [Table 7-1].
7.3.5.1.5 Lung Injury

HCA significantly reduced stretch induced lung injury compared to Normocapnia. HCA reduced the decrease in arterial oxygen tension over the course of the protocol, and paO₂ was significantly higher at all time points with HCA compared to Normocapnia [Table 7-1; Figure 7-4]. HCA significantly reduced alveolar-arterial oxygen gradient compared to Normocapnia at the end of the protocol [Table 7-1]. HCA significantly attenuated the decrease in static lung compliance compared to Normocapnia, with static lung compliance significantly higher in the HCA group compared to Normocapnia at the end of the protocol [Table 7-1; Figure 7-5]. HCA significantly reduced lung permeability, as evidenced by reduced bronchoalveolar protein concentrations [Figure 6]. Lung wet/dry weight ratios were higher with Normocapnia, but this difference was not statistically significant [Table 7-1].

7.3.5.1.6 Pulmonary inflammation and NF-κB activation

HCA significantly reduced BAL neutrophil counts [Figure 7-7], BAL interleukin-6 [Figure 7-9], BAL TNF-α [Figure 7-10], and BAL CINC-1 [Figure7-11], the homologue of the NF-κB dependent human cytokine interleukin-8 compared to Normocapnia. Cytoplasmic concentrations of the NF-κB inhibitor IκB-α were reduced to a similar extent in animals exposed to Normocapnia and HCA, compared to sham animals [Figure 7-12].

7.3.5.1.7 Histological injury

Quantitative stereological analysis demonstrated that HCA reduced the degree of histologic injury, as evidenced by an increased alveolar airspace fraction and a reduced tissue fraction with HCA, compared to animals exposed to Normocapnia [Figure 7-13]. Representative micrographs from an animal exposed to VILI under conditions of HCA and Normocapnia demonstrate greater loss of alveolar architecture and thickening of alveolar septae animals exposed to Normocapnia [Figure 7-14].
7.3.5.2 Effects of HCA in moderate VILI

Twelve animals were entered into this study. No animals were excluded prior to randomization, and all 12 animals were randomized to receive Normocapnia \( [n = 6] \) or HCA \( [n = 6] \). All animals in both groups survived the injury protocol [Table 7-2].

7.3.5.2.1 Baseline characteristics

There were no differences between the groups at baseline with regard to animal weight, MAP, \( p_aO_2 \), \( p_aCO_2 \), arterial pH, serum lactate and bicarbonate, and static compliance [Table 7-2].

7.3.5.2.2 Arterial CO\(_2\) tension and acid base

Arterial pH and \( PaCO_2 \) were similar in the Normocapnia and HCA groups at baseline [Table 7-2]. There was an initial rapid increase in \( PaCO_2 \) and decrease in pH in the HCA group following the induction of hypercapnia. At each hourly time point during the experiment \( PaCO_2 \) was higher and pH was lower in the HCA than in the Normocapnia group [Table 7-2; Figure 7-14; Figure 7-16]. There were no differences between the groups in serum bicarbonate or base excess at baseline or at the end of the protocol [Table 7-2]. In contrast, HCA did significantly attenuate the increase in serum lactate over the course of the protocol, and serum lactate was significantly lower at all time points, compared to Normocapnia [Table 7-2; Figure 7-17].

7.3.5.2.3 Hemodynamic profiles

Mean arterial pressure decreased significantly in both groups over time, but was not different between the groups [Table 7-2].

7.3.5.2.4 Lung injury

HCA significantly attenuated the decrease in arterial oxygen tension over the course of the protocol, and arterial \( PO_2 \) was significantly higher at 60, 120 and 180 minutes, compared to Normocapnia [Figure 7-18]. HCA reduced the alveolar-arterial oxygen gradient compared to Normocapnia at the end of the protocol, but this difference was not statistically significant [Table 7-2, Figure 7-19]. Static lung compliance decreased
significantly in both groups over the course of the protocol, but this decrement in compliance was not significantly altered by HCA [Table 7-2; Figure 7-20]. There was no difference in bronchoalveolar protein concentrations [Figure 7-21] or in lung wet/dry weight ratios between the groups at the end of the protocol [Table 7-2].

7.3.5.2.5 Pulmonary inflammation and NF-κB activation

HCA significantly reduced BAL neutrophil counts [Figure 7-22] compared to Normocapnia. HCA reduced serum concentrations of CINC-1 [Table 7-2]. In contrast, BAL concentrations of CINC-1, interleukin-6 and TNF-α [Table 7-2] were not different between the groups. HCA abolished the decrease in total and cytoplasmic IκB-α concentrations induced by high lung stretch, maintaining cytoplasmic IκBα concentrations at levels comparable to that seen in sham, non-ventilated animals [Figure 7-23; Figure 7-24]. HCA also reduced the nuclear p65 content compared to Normocapnia [Figure 7-25].
7.4 DISCUSSION

7.4.1 EFFECT OF HCA ON MODERATE AND SEVERE VILI

Hypercapnic acidosis reduced the severity of both moderate and severe VILI, however, the protective effect of hypercapnia was more obvious when lungs were exposed to severe mechanical stretch. In the severe VILI series HCA significantly attenuated the decrement in oxygenation, reduced lung permeability, alveolar neutrophil infiltration, and histologic injury. Lung inflammatory markers, such as BAL IL-6, TNF-α and CINC-1 were also significantly suppressed by HCA under severe VILI. In contrast, administration of CO₂ had less marked effect moderate VILI, evidenced by improvements only in three injury markers, such as oxygenation, BAL neutrophil content and serum lactate levels.

7.4.1.1 Effect of HCA on VILI – the role of injury severity

The first study demonstrating that HCA directly reduces VILI has been carried out by Broccard et al in an ex vivo rabbit lung model [88]. Isolated rabbit lungs were ventilated with either low PIP [15 cmH₂O] or gradually increasing PIP [20-25-30 cmH₂O] and exposed to normocapnia or HCA. The investigators found that HCA reduced microvascular permeability, lung oedema formation, and BAL protein content, however, the effect was clearly depended on the severity of the mechanical injury. HCA was protective at PIP: 30 cmH₂O, but not at lower PIPs, such as 20 or 25 cmH₂O. Of note, the pattern of lung injury seemed to be exponential, showing sudden increase when PIP was increased from 25 to 30 cmH₂O, further supporting the importance of threshold pressure in the development of VILI [See Chapter 6, Section 6.5.2.].

Subsequent study by Sinclair et al confirmed and extended these observations in an in vivo rabbit VILI model [89]. Although these studies provided the first evidence of the potential beneficial effect of HCA in VILI, the investigators used excessive injury to prove their hypothesis.

The question, how HCA affects VILI in a clinically more relevant ventilator strategy, has been subsequently explored by Laffey et al in an in vivo rabbit VILI model [125]. Ventilation with Vt 12ml/kg resulted in improved oxygenation and lung mechanics. Conversely, hypocapnic alkalosis worsened these parameters. Recently, Halbertsma et
*al*, reported that low \( V_t \) ventilator strategy \([V_t: 8 \text{ ml/kg}]\) with PEEP \([4 \text{cmH}_2\text{O}]\) for 2 h was associated with increased BAL neutrophil count and pro-inflammatory cytokines in *in vivo* mouse model compared to non-ventilated controls. Furthermore, administration of 2 or 4% \( \text{CO}_2 \) to the breathing circuit significantly attenuated mechanical stretch induced lung inflammation [180].

Most recently, *Pletekova et al* revisited the effect of HCA on inflammatory response in low and high \( V_t \) [12 vs. 45 ml/kg] ventilation in *in vivo* mouse model [138]. HCA significantly improved oxygenation, lung elastance, microvascular permeability and lung histology in animals ventilated with the high \( V_t \) group, but not in the low \( V_t \). This was associated with significantly reduced lung mRNA expression of COX2 and COX2 protein synthesis only in the high \( V_t \) group.

### 7.4.1.2 Effect of HCA on other ALI models – the role of injury severity

This phenomenon has been seen in other ALI models. Hypercapnic acidosis did not influence lung injury in mild *E.Coli* pneumonia model when \( 8.4 \times 10^8 \) CFU bacteria were instilled to rat lungs [141]. In contrast, increasing severity, i.e. administration of \( 5-6.4 \times 10^9 \) [130] or \( 2-3.2 \times 10^{15} \) CFU *E.Coli* [42], resulted in increasing protection against live bacteria induced ALI under hypercapnic conditions. Equally, in a systemic sepsis induced ALI model, hypercapnic acidosis was more protective when 50% CLP was applied as opposed to 37.5% CLP [131].

### 7.4.1.3 Conclusion

Our findings together with the above listed data are intriguing for several reasons: (1) HCA seems to be protective when the injury is severe. This is in line with the secondary analysis of the ARMA study by *Kregenow et al* [69]; (2) the effect of HCA in the context of less severe mechanical stretch induce injury is unclear and may relate to the fact that it is difficult to measure treatment effect when the difference between control condition and intervention is small; (3) however, our experiment provided another piece of evidence that the protective effect of HCA in the acute phase of VILI, even in the context of less severe injury is protective; (4) even though, *Kregenow et al* did not find any mortality benefit in the low \( V_t \) group, the above listed preclinical findings indicate that the biological effect of hypercapnia may have not been lost during lung
protective ventilation. It is a well established concept that protective lung ventilation in ARDS patients is able to generate regional alveolar overdistentions [52], therefore, it is important to investigate directly how permissive hypercapnia acts in this situation.

Our data reassures that HCA is protective when mechanical stretch induced injury is less severe.

7.4.2 HCA INHIBITS STRETCH INDUCED NF-κB PATHWAY

We have demonstrated that HCA reduced activation of NF-κB pathway in moderate VILI. This was evidenced by reduced production of NF-κB dependent cytokines in BAL, such as CINC-1, IL-6 and TNF-α. We also showed that HCA inhibited NF-κB activation, by maintaining the cytosolic and total cellular levels of the inhibitor IκB-α at similar levels to that seen in non-ventilated animals. In parallel, hypercapnia reduced p65 content in the nucleus, further supporting the fact that NF-κB signal transduction is attenuated by HCA.

7.4.2.1 HCA and NF-κB pathway - current state of knowledge

The central role of NF-κB in mediating the inflammatory response to mechanical stretch is well established in in vivo and in vitro experimental models [See Chapter 1, Section 1.3.2]. However, very little is known about how hypercapnic acidosis mediates its biological effects in the context of ALI. The first publication reporting the potential of HCA to attenuate endotoxin induced injury via an NF-κB dependent mechanism was published by Takeshita et al [190]. The authors demonstrated that HCA prevented IκB-α degradation in LPS stimulated pulmonary endothelial cells and this coexisted with decreased ICAM-1, IL-8 mRNA expression, cytokine and LDH release. This observation has been extended by our group demonstrating that HCA attenuated pulmonary epithelial wound healing via inhibition of IκB-α degradation [134]. Concurrently with our findings, others have suggested that the CO₂ molecule may directly inhibit the NF-κB pathway [135-137]. These studies showed that administering various concentration of carbon dioxide [5 - 20 %CO₂], facilitated the nuclear transport of IKKα, reduced IκB-α degradation in the cytoplasm, inhibited p65 nuclear translocation and the activation of the transcription complex in various LPS stimulated cell lines [135]. NF-κB pathway
inhibition via CO₂ resulted in the following biological effects: (1) reduced expression of pro-inflammatory genes [CCL2, ICAM1, TNF-α], while ant-inflammatory genes, such as IL10, were enhanced [135]; (2) CO₂ impaired host defence by inhibiting the induction of specific NF-κB dependent antimicrobial peptide genes in Drosophila [137]; (3) hypercapnia inhibited phagocytosis in macrophages stimulated with LPS [136]. Most recently, Wu et al demonstrated that HCA reduced the inflammatory response in the context of pulmonary ischaemia-reperfusion injury by inhibiting IκB-α degradation and NF-κB nuclear translocation in an ex vivo lung injury model [140].

Overall, these observations suggest that there may be a casual correlation between the effect of hypercapnia and the inhibition of the NF-κB pathway. Our results confirm and extend these findings. Although, there are several limitations to our experiments, this is the first time that it has been demonstrated in an in vivo VILI model that the anti-inflammatory effect of HCA is mediated via inhibition of the NF-κB pathway.

7.4.2.2 Limitation of the study

We demonstrated that HCA inhibited the NF-κB pathway in the moderate, but not in the severe VILI model. The explanations for this can be summarised as follows:

(1) It is important to notice, that in the severe VILI series the normocapnic animals died much earlier and at differing rate than in the moderate VILI series, therefore lung samples taken at the end of the experiments were from different time points and then pooled. This may explain, at least in part, why we could not detect significant difference in IκB-α degradation in animals exposed to more aggressive ventilation strategy.

(2) The other possibility may relate to the regulation of the NF-κB pathway. The key step for controlling NF-κB activity and signal responsiveness is the interaction between IκB-α and NF-κB [202]. Intracellular regulation of this step is very complex, however it is generally accepted, that the activated NF-κB increases the resynthesis of IκB-α proteins resulting in a negative feedback. The resynthetized IκB-α, at least partially and temporally, terminates the transcription by retaining the NF-κB dimers in the cytoplasm. Furthermore, others have suggested that IκB-α degradation and
resynthesis has a cyclic pattern and may result in oscillations in the temporal response in NF-κB signalling [334]. It has been reported both in *in vitro* and *in vivo* conditions that NF-κB translocation and IκB-α degradation occur very early in response to mechanical stretch, somewhere between the first 15-30 minutes, however, these intracellular events return close to the initial levels after 1-2 hours [95, 96, 98]. Similar NF-κB activation pattern has been described by others using different type of injuries, such as irradiation [192], ischaemia-reperfusion [213] or endotoxin [190]. With regard to our experiment it is possible that in the severe VILI series we missed the “window of opportunity” to detect the effect of HCA on IκB-α degradation. Conversely, it is also possible that our findings occurred by chance in the moderate VILI series.

(3) At last, we measured IκB-α and p65 levels in total lung tissue samples, which constitutes many different cell types. The interaction among cells and their individual response to mechanical stretch and HCA may introduce bias and may distort the information about the effect of hypercapnia in VILI. Also, the involvement of different organ systems, multiple cells and the presence of various cytokines further modifies the temporo-spacial pattern of NF-κB activity making the interpretation of such results difficult.

To circumvent the problems related to methodology and the complexity of the *in vivo* animal model, we sought to investigate the effect of HCA on NF-κB activation in separate *in vitro* experimental series of mechanical stretch induced VILI model. Ansari *B* demonstrated that cyclic mechanical stretch activated NF-κB in pulmonary epithelial cells, and this effect was attenuated by overexpression of the IκB-α transgene [335]. Both moderate [5%), and more severe [10%] HCA reduced stretch-induced NF-κB activation in parallel with reduced IL-8 production. Importantly, HCA inhibited cellular injury and enhanced cell survival under conditions of prolonged cellular stretch. Although, this obviously does not fully model the complexity of the *in vivo* situation, it confirms our findings, and provides another piece of evidence on the biological mechanism of HCA in VILI.
7.5 SUMMARY

Our findings supports the hypothesis that HCA reduces inflammatory response and beneficial in the acute phase of VILI. We also provide additional evidence that NF-κB pathway inhibition via HCA mediates the protective effects of HCA in the setting of stretch induced lung injury. Overall, these data extend our understanding of the mechanism of action of hypercapnia and provides the first evidence on the relationship between HCA and NF-κB pathway in a relevant in vivo pre-clinical model.

7.6 CONCLUSIONS

1. HCA protects against moderate and severe VILI in an in vivo rodent model.

2. The protective effect of HCA in VILI appears to be mediated via NF-κB pathway inhibition, evidenced by reduced IκB-α degradation in the cytoplasm and reduced nuclear p65 translocation in lung samples.

3. The protective effect of HCA depends on injury severity. Severe VILI allowed us to demonstrate the physiological, inflammatory and histological changes caused by HCA, while moderate VILI model was suitable to demonstrate more subtle intracellular changes, such as inhibition of the NF-κB pathway by HCA.
### Table 7-1

Effect of HCA on animals exposed to severe VILI. Summary of survival and physiological variables. Data are expressed as mean ±S D. Final data is data collected upon completion of the experimental protocol.

* Significantly different from Normocapnia [P <0.05]

† Significantly different from Normocapnia [P <0.01]

‡ Significantly different from Baseline [P <0.05]

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normocapnia</th>
<th>HCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
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<tr>
<td>Animal Weight [g]</td>
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<td>392 ± 25</td>
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<td>Animal survival [%]</td>
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<td>6/7 [86%]</td>
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<td>Duration of survival [minutes]</td>
<td>166 ± 69</td>
<td>229 ± 28*</td>
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<td>Serum Bicarbonate [mmol/l]</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.3 ± 1.3</td>
<td>23.3 ± 0.9</td>
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<tr>
<td>Final</td>
<td>16.7 ± 4.4†</td>
<td>16.9 ± 2.4‡</td>
</tr>
<tr>
<td>Base Excess [mmol/l]</td>
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</tr>
<tr>
<td>Baseline</td>
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<tr>
<td>Final</td>
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<td>-6.8 ± 3.0</td>
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<tr>
<td>Mean Arterial Pressure [mmHg]</td>
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</tr>
<tr>
<td>1 Hours</td>
<td>119 ± 23‡</td>
<td>95 ± 12**‡</td>
</tr>
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<td>2 Hours</td>
<td>84 ± 30‡</td>
<td>102 ± 17‡</td>
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<td>3 Hours</td>
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<td>4 Hours</td>
<td>68 ± 12‡</td>
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<td>Arterial O₂ tension [kPa]</td>
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<td>Final [FiO₂ 0.3]</td>
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<td>15.1 ± 3.2‡‡</td>
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<td>[FiO₂ 1.0]</td>
<td>21.2 ± 22.6</td>
<td>50.2 ± 22.6*</td>
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<tr>
<td>Alveolar-arterial Oxygen Gradient [kPa]</td>
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<td>0.70 ± 0.04*‡</td>
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<tr>
<td>Wet: Dry Weight Ratio</td>
<td>7.4 ± 1.4</td>
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Figure 7-1 Arterial pH over time in animals exposed to severe VILI. Data are expressed as mean±SD. * Significantly different from Normocapnia [P <0.05].

Figure 7-2 Arterial pCO₂ levels over time in animals exposed to severe VILI. Data are expressed as mean±SD. * Significantly different from Normocapnia [P <0.05].
Figure 7-3 Serum lactate levels over time in animals exposed to severe VILI. Data are expressed as mean ± SD. * Significantly different from Normocapnia [P < 0.05].

Figure 7-4 Arterial pO₂ levels over time in animals exposed to severe VILI. Data are expressed as mean±SD. * Significantly different from Normocapnia [P < 0.05].
Figure 7-5 Lung elastance in animals exposed to severe VILI. Data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [P <0.05].

Figure 7-6 Bronchoalveolar lavage [BAL] protein concentration in animals exposed to severe VILI. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [p <0.05].
Figure 7-7 Bronchoalveolar lavage [BAL] neutrophil cell count in animals exposed to severe VILI. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [p <0.05].

Figure 7-8 Bronchoalveolar lavage [BAL] IL-6 levels in animals exposed to severe VILI. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [p<0.05].
Figure 7-9 Bronchoalveolar lavage [BAL] TNF-α levels in animals exposed to severe VILI. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [p<0.05].

Figure 7-10 Bronchoalveolar lavage [BAL] CINC-1 levels in animals exposed to severe VILI. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia [p<0.05].
Figure 7-11 Histogram representing mean±SD lung tissue cytoplasmic IkB-α concentrations in severe VILI. Cytoplasmic IkB-α concentrations were reduced to a similar extent with both Normocapnia and HCA, compared to sham animals. Representative western blot of lung tissue cytoplasmic IkBα in sham animals, animals exposed to VILI under HCA conditions and under normocapnic conditions. The abbreviations used are as follows: Sham [non-ventilated] animals; NC, Normocapnia; HCA, Hypercapnic acidosis.

† Significantly different from sham [P <0.05].
Histogram representing stereologic assessment of the extent of histologic injury in severe VILI. Tissue volume fraction represents all alveolar tissue including epithelium, endothelium, connective tissue and inflammatory cells. Extra-acinar fraction means bronchial or vascular space, and airspace describes the gaseous component of the lung. * Significantly different from Normocapnia [p<0.05].

Representative photomicrograph of lung tissue from an animal subjected to severe VILI under HCA conditions or Normocapnia.
<table>
<thead>
<tr>
<th>Variable</th>
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<th>HCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
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</tr>
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<td>6/6 (100)</td>
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<tr>
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<td>3 Hours</td>
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<td>7.16 ± 0.03‡‡</td>
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<td>4 Hours</td>
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<td>7.14 ± 0.03‡</td>
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<td>Arterial CO₂ tension [kPa]</td>
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<td>1 Hours</td>
<td>4.7 ± 0.8</td>
<td>8.8 ± 1.3‡‡</td>
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<td>5.4 ± 0.4‡</td>
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<td>4 Hours</td>
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<td>Serum Bicarbonate [mmol/L]</td>
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<td>1 Hours</td>
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<td>2 Hours</td>
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<tr>
<td>4 Hours</td>
<td>4.9 ± 2.8‡</td>
<td>3.0 ± 0.8*</td>
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<tr>
<td>Mean Arterial Pressure [mmHg]</td>
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<tr>
<td>Baseline</td>
<td>153 ± 15</td>
<td>139 ± 13</td>
</tr>
<tr>
<td>1 Hours</td>
<td>118 ± 19‡</td>
<td>117 ± 14‡</td>
</tr>
<tr>
<td>2 Hours</td>
<td>137 ± 20‡</td>
<td>135 ± 21‡</td>
</tr>
<tr>
<td>3 Hours</td>
<td>129 ± 22‡</td>
<td>132 ± 22‡</td>
</tr>
<tr>
<td>4 Hours</td>
<td>112 ± 21‡</td>
<td>126 ± 22‡</td>
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<tr>
<td>Arterial O₂ tension [kPa]</td>
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</tr>
<tr>
<td>Baseline</td>
<td>18.3 ± 0.8</td>
<td>18.8 ± 1.3</td>
</tr>
<tr>
<td>1 Hours</td>
<td>16.5 ± 1.1‡</td>
<td>18.4 ± 1.3*</td>
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<tr>
<td>2 Hours</td>
<td>14.9 ± 1.3‡</td>
<td>17.7 ± 1.7‡</td>
</tr>
<tr>
<td>3 Hours</td>
<td>13.2 ± 1.5‡</td>
<td>16.2 ± 1.2‡</td>
</tr>
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<td>4 Hours [FiO₂ 0.3]</td>
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<td>[FiO₂ 1.0]</td>
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<tr>
<td>Baseline</td>
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<tr>
<td>Final</td>
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<td>0.66 ± 0.05†</td>
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<td>Wet: Dry Weight Ratio</td>
<td>5.9 ± 1.7</td>
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<tr>
<td>BAL CINC-1 concentration [pg/ml]</td>
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<td>2956 ± 1078</td>
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<td>Serum CINC-1 concentration [pg/ml]</td>
<td>10188 ± 5770</td>
<td>3779 ± 2308*</td>
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<td>BAL TNF-α concentration [pg/ml]</td>
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<td>130 ± 56</td>
</tr>
<tr>
<td>BAL IL-6 concentration [pg/ml]</td>
<td>274 [125, 430]</td>
<td>318 [289, 433]</td>
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</tbody>
</table>

Table 7-2 Effect of HCA on animals exposed to moderate VILI. Summary of survival, physiological and inflammatory markers. Data are expressed as mean±SD or as median [Interquartile range]. Final data is data collected upon completion of the experimental protocol. * Significantly different from Normocapnia (P <0.05). † Significantly different from Normocapnia (P <0.01). ‡ Significantly different from Baseline (P <0.05).
Figure 7.14 Arterial pH over time in animals exposed to moderate VILI. * Significantly different from Normocapnia\(p < 0.05\).

Figure 7.15 Arterial pCO\(_2\) over time in animals exposed to moderate VILI. * Significantly different from Normocapnia\(p < 0.05\).
Figure 7-16 Graph representing mean±SD arterial lactate levels at baseline and over the course of the protocol in moderate VILI. Increase in arterial lactate levels was significantly greater in animals exposed to Normocapnia. * Significantly different from Normocapnia [p<0.05].

Figure 7-17 Graph representing mean±SD arterial oxygen pressures at baseline and over the course of the protocol in moderate VILI. The decrement in arterial oxygen tensions was significantly greater in animals exposed to Normocapnia. * Significantly different from Normocapnia [p<0.05].
Figure 7-18 Graph representing mean±SD Alveolar-arterial oxygen gradient [DAaO2] in moderate VILI. HCA reduced DAaO2, however there was no significant difference compared to Normocapnia.

Figure 7-19 Lung elastance in animals exposed to moderate VILI. Data is data collected upon completion of the experimental protocol.
Figure 7-20 Histogram representing mean±SD bronchoalveolar lavage protein [BAL] concentration in moderate VILI. HCA reduced BAL protein compared to Normocapnia, however the difference was not significant.

Figure 7-21 Histogram representing mean±SD bronchoalveolar lavage [BAL] neutrophil counts in moderate VILI. * Significantly different from Normocapnia [p<0.05].
Figure 7-22. Histogram representing mean±SD total lung tissue IκBα content in moderate VILI. Total IκBα was significantly higher in animals exposed to HCA compared to Normocapnia. *Significantly different from Normocapnia [p<0.05].

Figure 7-23. Histogram representing mean±SD densitometric measurements of lung tissue cytoplasmic IκBα content from Western Blots in moderate VILI. HCA abolished the decrease in cytoplasmic IκBα content induced by high lung stretch. Representative western blot of lung tissue cytoplasmic IκBα in sham animals, animals exposed to VILI under HCA conditions and under normocapnic conditions. The abbreviations used are as follows: Sham [non-ventilated]
animals; Normo, Normocapnia; HCA, Hypercapnic acidosis. * Significantly different from HCA [p<0.05].

Figure 7-24 Histogram representing mean±SD of p65 nuclear extracts from lung tissue in moderate VILI. * Significantly different from Normocapnia (P<0.05).
CHAPTER 8 : GENERATION OF RECOMBINANT AAV VECTOR CONTAINING IκB-α-SUPER-REPRESSOR TRANSGENE AND NULL VECTOR

8.1 ABSTRACT

Background: Adeno associated viruses [AAVs] have been widely used in both preclinical and clinical studies as a vector delivery system to treat pathological conditions. AAV vectors are safe, non toxic, not carcinogenic, provide stable and prolonged transgene expression, and have diverse tissue tropism. The main disadvantages of AAV vectors include the possibility to trigger exaggerated host immune response to viral capsid leading to reduced transgene expression or impeding the effectiveness of repeated transgene administration. In addition, AAV vectors have low packaging capacity limiting their use for relatively smaller transgenes. In this chapter we wish to describe the manufacturing of rAAV6-IκBα-super-repressor [rAAV6-IκBα-SR] transgene for later experiment in order to investigate the potential of NF-κB pathway inhibition via gene transfer in the development of VILI.

Methods: A two-plasmid system was used to transfect immortalized cell lines [human embryonic kidney 293T cells] to generate recombinant AAV6 particles containing IκBα-SR transgene or Null vector [rAAV6Rep/Cap]. One of the AAV plasmid contained the transgene of interest [pAAV-IκBα-SR], the other plasmid carried DNA sequences for Rep genes [genes responsible for replication], Cap genes [genes decoding viral capsid] and adenoviral [Ad] helper genes to aid replication and packaging. Purification process included: Iodixanol gradient centrifugation, ion exchange chromatography, desalting and concentration using serial washes and centrifugation. Virus titre was determined using Real time-PCR.

Results: Titres achieved for rAAV6-IκBα-SR and rAAV6-Null transgene were: 10^{7-9} particle/μl. These titres were lower that have been previously described in the literature.

Conclusion: The protocol described below resulted in sufficient amount of viral products to carry out in vivo experiments. The main hurdle to efficient rAAV manufacturing may have been related to transfection of an NF-κB pathway inhibitor
which is a large transgene, and possibly to the low recovery of viral particles during purification process.
8.2 INTRODUCTION

8.2.1 GENERAL OVERVIEW

AAVs have been successfully used as carrier particles to deliver genetic materials both in preclinical and clinical studies [218, 220, 225]. This is due to their safety features and effectiveness in delivering transgenes to wide range of organs. Overall, these characteristics include: (1) AAVs are non-pathogenic and up to date AAVs have not been described as pathogenic organisms in any human diseases [220]. (2) AAVs have been shown to be the safest viral vectors carrying almost negligible risk for carcinogenesis [220]. (3) AAVs provide stable and long term gene expressions in various organs [336]. (4) The existence of multiple virus serotypes provides an excellent opportunity to develop organ specific vector systems [282]. The main drawback of AAV vectors are their immunogenicity. A large portion of the human population has been infected with AAV2, possibly earlier in childhood, and around 90% of the population is seropositive to AAV2 [337]. Administration of AAV vectors therefore may trigger the already primed immune system and could lead to limited transgene transduction [220, 221]. Other disadvantages of AAVs include their small size, and relatively low packaging capacity, which limits their use to deliver transgene constructs at a size less than 4.7 kilo basepairs [kbp].

8.2.2 AAVS - LESSONS FROM CLINICAL TRIALS

The above listed characteristics of AAVs underpin the rationale to utilize AAVs for gene therapy in humans. This is supported by the fact that 36 clinical trials involving AAV-vectors have been approved for treating various human diseases [www.clinicaltrials.gov].

8.2.2.1 Cystic Fibrosis

Cystic fibrosis [CF] was one of the first diseases which were treated with AAV mediated gene transfer. Early trials demonstrated that AAV mediated gene transfer did not carry any toxicity, was safe, and was associated with minimal host immune response [227-229]. Although gene delivery was successful, pulmonary function did not improve in patients receiving gene therapy in two subsequent studies [226, 338]. This may have been related to more active epithelial shedding in the airways of CF patients. This
highlights the importance of biological barriers to gene transfer and the potential need of repeated administrations to achieve therapeutic benefits.

### 8.2.2.2 Haemophilia B

Clinical trials to treat Haemophilia B illustrate well how exploiting various vector delivery routes, serotypes and molecular engineering can overcome difficulties related to gene transfer. The first phase I study used intramuscular delivery of AAV2 vectors to replace dysfunctional factor IX [FIX] without any success [339]. Since FIX is synthesised in the liver, subsequent studies targeted this organ via the hepatic artery. Although it was possible to achieve therapeutic levels in FIX at the highest dose range, most of these patients developed elevated liver transaminase levels as a result of cytotoxic T-cell response against transduced hepatocytes [340]. This was clearly related to the AAV2 capsid. These findings led to the generation of more advanced transgene construct with higher expression profile, and in order to circumvent the possibility of humoral immunity to AAV, AAV2 was pseudotyped with a capsid of serotype 8 [AAV8/2] [341]. Since AAV8 has lower sero-prevalence in humans than AAV2 and higher tropism to the liver, the AAV-transgene construct was successfully delivered via peripheral intravenous injection [341].

### 8.2.2.3 Parkinson’s disease

In Parkinson’s disease, gene delivery includes three major strategies: (1) introduction of dopamine-synthesizing enzyme genes; (2) protection of nigrostriatal pathways via transduction of a neurotrophic factor to dopaminergic neurons; (3) modulation of neural activity with vectors expressing glutamic-acid-decarboxylase, a key enzyme required for the synthesis of the inhibitory transmitter GABA [342, 343]. These approaches indicate that the main target in Parkinson’s disease is to improve a “loss of function” rather than cure the primary lesions, i.e. neuronal degeneration.

### 8.2.2.4 Conclusion

Experiences from clinical trials using AAV vector systems can be summarized as follows: (1) AAVs can be used to treat single gene defects [CF, Haemophilia B] and also to modify cell function [neurodegenerative diseases] in order to achieve optimal organ function; (2) certain vector delivery route can be used for more targeted and less
immunogenic gene transfer; (3) modifications in transgene and viral capsid composition can lead to better tissue tropism, higher expression efficiency and reduced immune response.

8.2.3 AAV-STRUCTURE

8.2.3.1 Wild type AAV2 structure

Adeno associated viruses are one of the smallest viruses, consisting of a non enveloped capsid [22-25 nm diameter] that packages a single stranded DNA [ssDNA] [220]. It is well described that active AAV production requires helper viruses. In the absence of helper virus, AAV integrates into the human genome and remains in a dormant form. When a latently infected cell is super-infected with a helper virus, AAV gene expression becomes activated followed by replication, packaging and cell lysis, resulting in the release of newly synthesised virions.

Recombinant AAV [rAAV] vectors have been generated based on the structure of the AAV2 serotype [225]. The AAV2 genome is linear, ssDNA of 4.7 kbp. At the two ends of the DNA are 145 base pair [bp] inverted terminal repeat [ITR] segments with palindrome sequences [first 125 bp] folding up and forming a T shaped structure. The main biological roles of the ITR segment are: (1) coordination of DNA replication; (2) genome packaging; (3) transcription; (4) site specific integration into the chromosome 19; and (5) negative regulation under non permissive conditions. The genome also consists of two open reading frames [ORFs] termed Rep, and Cap genes, which encode non-structural and structural proteins. The Rep gene produces four proteins [Rep78, Rep68, Rep52, Rep40] which are responsible for DNA replication and regulation independently from the presence of helper virus. The Cap gene produces three viral capsid proteins [VP1, VP2, VP3]. The final capsid comprises 60 viral capsid proteins arranged into an icosahedral structure. Adenoviral helper genes that are essential for AAV replication and are not present in the wild type AAV genome are identified as: E1a, E1b, E2a, E4 and VA RNA [220, 225].

8.2.3.2 Recombinant AAV

The structure of rAAV containing specific transgenes derived from the previously described AAV2 structure. In these vectors the Rep and Cap genes are replaced with a
gene expression cassette of interest [Figure 8-1 A-C]. In our experiments we used IκB-α-SR transgene co-expressed with GFP under the control of a CMV promoter inserted into an AAV plasmid. The structure of the complete pAAV-MCS-IκBαSR-GFP is described in [Figure 8-1 D]. Our final expression plasmid was circular, containing: (1) pUC ori sequence providing stable and high plasmid copy number following bacterial transformation, (2) an ampicillin resistance gene ensuring that only bacterial cells which take up the plasmid survive during bacterial propagation. This allows the selection of bacteria containing the plasmid from bacteria which do not have the plasmid, (3) f1 origin sequence, responsible for single stranded DNA replication, (4) Multiple cloning site [MCS] containing the IκBα-SR gene and GFP gene, (5) other structures of AAV including ITRs, CMV promoter, β-globin intron, hGH pA. We also used a second helper plasmid containing the Rep/Cap genes in association with the necessary adenoviral helper genes [Figure 8-1E].

8.2.3.3 AAV serotypes

The main advantage of large number of various AAV serotypes is the possibility to achieve tissue specific gene transduction. More than 10 AAV serotypes and more than 100 AAV variants have been identified over the last 30 years [220, 282].

The majority of preclinical and clinical studies have used AAV2 due to its reliable and broad tissue tropism. However, generally AAV2 seems to be moderately efficient when it is compared to other AAVs, such as AAV9 [the most efficient, but less tissue specific AAV]. Recently, Zincarelli et al analysed the expression profile, kinetics and tissue tropism of AAV1-9 in transgenic mice [234]. The group reported that the lowest tissue expression was observed with AAV2, 3, 4 and 5, while AAV1, 6 and 8 had moderate, and AAV 7 and 9 had high expression profile. Gene expression kinetics was rapid in AAV1, 6, 7, 8 and 9 and slow in the other serotypes. Hierarchy of tissue tropism of various serotypes in major organs have been reported by many groups. However, the results are not concordant. This may be related to the animal species used in the experiments, to the animal individual antibody profile [differences in environmental factors lab to lab], and also to other factors determining tissue tropism, such as cellular uptake, intracellular processing of the viral particles, nuclear delivery of the viral
genome, uncoating and viral DNA synthesis[221, 234, 282, 283, 344]. In general, lung tissue tropism is most favourable for AAV4, 5, 6 and 9.

8.2.3.4 AAV6 serotype

Halbert et al systematically investigated the potential of the AAV6 serotype to transduce both lung airway and alveolar epithelial cells [283, 344]. The group demonstrated that AAV6 serotype is superior to AAV2, reaching as high as 80% transduction rate in lung tissue. Transduction efficiency was further improved by using a high efficiency promoter for transgene expression. Others showed that AAV6 serotype has significantly higher lung tissue tropism and transduction efficiency in mice compared to AAV1, 2, 5, 7, 8 and 9 [345]. In addition, our group also have demonstrated, in rats, that transduction of EC-SOD transgene using recombinant AAV6 vector to injured lung tissue was superior compared to rAAV5 vector [unpublished data].

8.2.4 JUSTIFICATION TO EMPLOY RECOMBINANT AAV6 VECTOR SYSTEM

The rational to utilize recombinant AAV6 vector to deliver transgene to lung tissue in our later experiments was based on the following observations: (1) AAVs have been successfully delivered to lung tissue in clinical studies; (2) AAVs have been used successfully to modify cell function in clinical trials, a feature which can be exploited to modulate inflammatory response in ALI; (3) AAV6 has excellent lung tissue tropism; (4) our group has been successfully delivered rAAV6-transgene construct to injured lung in in vivo rat model of LPS induced ALI.

In the present chapter, we describe the process of AAV6-\(\text{I} \kappa \text{B}\alpha\)-SR production to generate high scale viral vector products for subsequent in vivo experiments. Our aim was to achieve a \(10^{13-14}\) viral particle/\(\mu\)l stock in order to demonstrate the effect of \(\text{I} \kappa \text{B}\alpha\) inhibition on ventilation induced lung injury.
A. Wild type AAV genome

B. Modified AAV genome containing transgene and wild type ITRs

C. Expression cassette

D. AAV-MCS-transgene/GFP plasmid

E. Helper plasmid
**Figure 8-1** Schematic descriptions of plasmid constructs

**A: Wild type AAV genome.** The Rep and Cap genes are flanked by ITRs [inverted terminal repeats]. P5 [Rep78/68], P19 [Rep 52/Rep40] and P40 [VP1/VP2/VP3] represent specific promoters responsible for the initiation of the actual Rep and Cap gene transcription.

**B: Modified AAV genome containing transgene and wild type ITRs.** Rep and Cap genes are replaced with a promoter-transgene construct.

**C: Expression cassette.** CMV promoter: cytomegalovirus promoter ensuring constitutive transgene expression once the transgene is incorporated in the host genome. \( \beta \) globin intron: allows higher transgene expression and mRNA splicing. EcoR1 and Sal1: restriction enzyme sites. \( \kappa B \alpha - SR \): \( \kappa B \alpha \) super-repressor transgene. IRES: internal ribosome entry site: Regulates the transcription of mRNA coding for \( \kappa B \alpha - SR \) and GFP. GFP: green fluorescent protein. Poly A tail: long chain of adenine nucleotides that is added to a messenger RNA [mRNA] molecule during RNA processing to increase the stability of the molecule.

**D: AAV-MCS-transgene/GFP plasmid.** The transgene is inserted into the multiple cloning sites [MCS]. The plasmid also contains f1ori: starting point of DNA replication. This sequence facilitates the replication of the plasmid in bacteria. Ampicillin: ampicillin resistance gene enables the survival only bacterias which contain the plasmid of interest during virus production. pUCori: it determines the vector copy number. pUCori sequence provides high-copy number, between 150 and 200 copies/cell, and provides greater stability during cell division, an important feature for viral production.

**E: Helper plasmid.** We used a helper plasmid encoding Rep and Cap [for AAV6 capsid serotype] DNA sequences linked with adenovirus helper genes [E2-VA-E4]. P5, P19, P40 are promoter sites to start specific gene expression decoding Rep/Cap proteins.
8.3 MATERIALS AND METHODS

The pAAV-\textit{IκB}α-\textit{SR}-GFP plasmid was kindly donated by Dr. Ralf Zwacka [NUIG, REMEDI]. \textit{IκB}α-\textit{SR} is a mutant form of \textit{IκB}-\textit{α}, in which serine residues 32 and 36 are mutated to alanines. \textit{IκB}α-\textit{SR} cannot be phosphorylated by the IKK complex, rendering it resistant to degradation.
### 8.3.1 VECTOR PRODUCTION

The provided flow chart summarizes the main steps of viral production described in the present chapter [Figure 8-2].

**Bacterial transformation**

*E. Coli* + plasmid [IκBα-SR,Rep/Cap]

*E. Coli*, containing the plasmid of interest, plated in Agar plates for growing

Picking colonies

Giga prep of plasmid to increase the stock

Quantification of plasmid DNA

Transfection of 293T HEK cells with using Jet PEI, pAAV-IκBαSR, pRep/Cap6

Incubation for 48 h

Harvesting and cell lysis by 3 freeze-thaw cycle

Endonuclease treatment

Purification by Iodixanol gradient

Ion exchange affinity chromatography

Concentration and desalting

Quantification of rAAV titre by Real Time PCR

**Figure 8-2** Simplified overview of steps of AAV production applied in our experiments. For viral products containing IκBα-SR-GFP transgene, two plasmids were used [pAAV-IκBα-SR-GFP and pRep/Cap6]. For viral products without transgene [Null] only pRep/Cap plasmid was used during transfection.
8.3.2 TRANSFORMATION OF COMPETENT BACTERIAL CELLS

Bacterial transformation is a widely used method to generate large amount of genetic material. This step was used to generate more IκBα-SR and AAV6-Rep/Cap plasmid for transgene production. [Figure 8-2].

Chemically competent bacterial cells that are able to take up plasmid DNA can be produced in the laboratory by suspending the cells in calcium chloride. Because both the DNA and the bacterial cell surface are predominantly negatively charged, the presence of positively charged calcium ions facilitates the attachment of the plasmid DNA to the cell membrane. Subsequently, the cells are exposed to heat-shock in a water bath which opens pores of the cell membrane and allowing the entry of plasmid DNA into the bacterium. As bacteria are dividing, the plasmid DNA together with the bacterial genome replicates and the number of plasmid DNA copies increase exponentially over time.

We used One Shot®TOP10F competent E. Coli cells [Invitrogen, #C3030-03]. For each plasmid, 50 μl of competent E. Coli suspension [1 vial] was thawed from -80°C freezer on ice. 1-5 μl of plasmid stock [pAAV-IκBα-SR, pRep/Cap6] was then added to the thawed cell suspension and tapped gently to ensure thorough mixing. Pipetting up and down was avoided as competent cells are sensitive to mechanical lysis. The mixture was incubated on ice for 30 minutes and subsequently exposed to heat shock for 30 seconds in a 42°C water bath. It is important to know that when E. Coli are subjected to 42°C heat, a set of genes are expressed which helps the bacteria in surviving at such temperatures. At temperatures above 42°C, the bacteria’s ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria die. Following the heat shock treatment, the cell suspension was placed on ice for 2 minutes before addition of 250 μl pre-warmed [room temperature] SOC medium [Super Optimal Broth with catabolite repression] to each vial. The vials were then placed in a rotary-shaker incubator for 1 hour at 225 rpm. This allowed the cells to recover after the heat shock. 50 μl of the cell suspension was aseptically spread on ampicillin supplemented [100μg/ml] Luria Berani [LB] agar [1% w/v] plate. The plates were then incubated at
37°C for 18 hours in an inverted position [to avoid condensation dripping onto the agar plate].

8.3.3 GENERATION OF GIGA PREPARATION OF PLASMID DNA

*E. Coli* colonies were picked up from LB [Difco™ LB broth, Becton Dickinson, Ireland] plates containing either pAAV6-Rep/Cap or pAAV-IκBα-SR plasmid and added to 15 ml tubes with 500 μl ampicillin containing [100 μg/ml] LB broth. The tubes were then placed in the rotary-shaker at 37°C at 225 rpm for 2 hours. Subsequently, the 500 μl bacterial suspension was added to a larger stock which was prepared by mixing a total volume of 2500 ml LB broth with 250 μl ampicillin stock [1 mg/ml]. The flasks were then placed in the rotary-shaker at 37°C at 225 rpm for 16-18 hours. Plasmid DNA was subsequently extracted from the bacterial culture using the Qiagen™ Qiprep© GigaPrep kit [See Chapter 3, Section 3.6.3.2]. The pellet was resuspended in dH₂O and its concentration was determined using spectrophotometry.

8.3.4 QUANTIFICATION OF PLASMID DNA

Quantification of the plasmid DNA obtained from the Gigapreps was determined by the use of a NanoDrop™ 1000 spectrophotometer. The concentration of the plasmid DNA was expressed as micrograms per milliliter [μg/ml]. This is described in more details in the Materials and Methods chapter under Section 3.6.3.3.

8.3.5 TISSUE CULTURE TECHNIQUE FOR VIRAL PRODUCTION

Human embryonic kidney cells [293-T] cells were used for viral production. 293-T cells are very easy to grow, and transfect readily. More detailed description is provided in Chapter 3 under Section 3.6.3.4-3.6.3.7.

8.3.6 CALCULATION OF PLASMID CONCENTRATION FOR CO-TRANSFECTION INTO 293-T CELLS TO GENERATE AAV CONTAINING IκB-α-SR TRANSGENE

For each transfection, a 1:1 molar ratio of the pAAV6Rep/Cap and plasmid coding IκB-α-SR transgene [pAAV- IκB-α-SR] were combined for a total of 1000 μg of plasmid DNA per forty plates [250μg/10 plates]. The relative molecular weight of each plasmid determined the optimal ratio into which the 1000 μg of total DNA plasmid were divided.
Example:

\[
\text{Plasmid ratio} = \frac{[\text{pRap}/\text{Cap6}]}{[\text{pAAV}_k\text{B}_\alpha - \text{SR}]} = \frac{22000 \text{ bp}}{4500 \text{ bp}}
\]

Total DNA plasmid for 40 plates = 1000 \( \mu \)g

\[
\text{Rap/Cap6 plasmid} = \frac{22000 \times 1000}{(22000 + 4500)} \approx 830 \mu \text{g}
\]

\[
\text{AAV}_k\text{B}_\alpha - \text{SR plasmid} = \frac{4500 \times 1000}{(22000 + 4500)} \approx 170 \mu \text{g}
\]

8.3.7 CO-TRANSFECTION OF REP/CAP6 PLASMID AND TRANSGENE PLASMIN INTO 293T CELLS

One day prior to transfection, 8 \( \times \) 10\(^6\) cells were seeded into each 150 mm plates in 20 ml of DMEM. After 24 hours, cells were approximately 50-70% confluent. We used 40-80 plates for each transfection experiment. Medium was changed prior to each transfection [See Chapter 3, Section 3.6.3.4-7].

We used a jetPEITM as a transfection agent [Autogen Bioclear LTD]. jetPEITM is a positively charged polymer that is able to compact and surround the plasmid DNA, hence improving the adherence of DNA to the negatively charged proteoglycan molecules at the cell surface and facilitating cell entry. The plasmid DNA is then internalized by endocytosis. Inside the cell the endosome ruptures and the plasmid DNA is ready for nuclear transport and subsequent transcription. One of the most important factors determining successful transfection is the overall ionic charge of the jetPEI/DNA complexes. This ionic balance is defined as the N/P ratio; \( N \) representing the nitrogen residues [positive charges] and \( P \) the phosphate residues [negative charges] in the complex. To achieve positively charged complexes, \( N/P > 3 \) is recommended. The following equation was used to calculate N/P ratio:

\[
N/P \text{ ratio} = \frac{7.5 \times \mu l \text{ jetPEI}}{3 \times \mu g \text{ DNA}}
\]

[7.5: concentration of nitrogen residues in jetPEI; 3: nmols of phosphate per \( \mu \)g in DNA].
One of the advantage of jetPEI compared to other transfection methods is that it is less cytotoxic, however high N/P ratios (>7.5) have been associated with significant cell death. This can be avoided by reducing exposure time to the transfection agent. In order to optimize transfection of 293-T cells, three small experiments were carried out identifying the optimal time and N/P ratio for transfection.

8.3.8 OPTIMIZATION OF 293T CELL TRANSFECTION USING JETPEI

Our aim was to optimize transfection with jetPEI in order to achieve at least 50% transfection rate after 48 hours. We looked at the effect of exposure time at three different N/P ratio on transfection rate.

8.3.8.1 Experiment I. – Effect of 48h exposure time on transfection rate.

Experimental set up: A three group experiment was carried out including 20 x 150 mm plates [total of 60 plates] in each group. Group I was transfected with a N/P ratio 2.5 [N/P:2.5]; Group II with N/P ratio 5 [N/P:5]; and Group III with N/P ratio 7.5 [N/P:7.5]. After 48 hours, cells were examined with confocal fluorescence microscopy [Table 8-1].

<table>
<thead>
<tr>
<th>Group</th>
<th>N/P ratio</th>
<th>DNA (µg)/20 plates</th>
<th>jetPEI (µl)/20 plates</th>
<th>Transfection time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.5</td>
<td>500</td>
<td>500</td>
<td>48</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>500</td>
<td>1000</td>
<td>48</td>
</tr>
<tr>
<td>III</td>
<td>7.5</td>
<td>500</td>
<td>1500</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 8-1 Each group consisted of 20 plates. The total amount of plasmid and jet PEI required for different N/P ratio is summarized in the table. Transfection time [incubation] was 48 hours.

Results: After 48 hours, cells were confluent, alive and transfected in Group I, however, transfection was less than 50%. In Group II, and III, cells were dead in spite of good transfection. These results indicated that 48 hours exposure to jetPEI with medium and high N/P ratio is cytotoxic [Figure 8-3].
Figure 8-3 A: 293T cells after 48 hours of transfection. Cells were around 70-80% confluent confirmed with light microscopy [10xmagnification]. B: Fluorescent picture of the same cell population. Cells containing IκBα-SR-GFP gene were fluorescent, representing the cell population which was successfully transfected [10x magnification].

8.3.8.2 Experiment II. – Effect of 4h exposure time on transfection rate.

Experimental set up: In this experiment we exposed 293T cells to transfection mix for 4 hours, followed by removal of the transfection mix and replacement with fresh DMEM medium. Three experimental groups included transfection with N/P: 2.5 [Group I], N/P: 5 [Group II], N/P: 7.5 [Group III]. Each group included 20 x 150 mm plates [total of 60 plates]. After 48 hours, cells were examined with confocal fluorescence microscopy [Table 8-2].

<table>
<thead>
<tr>
<th>Group</th>
<th>N/P ratio</th>
<th>DNA (µg)/20 plates</th>
<th>jetPEI (µl)/20 plates</th>
<th>Transfection time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.5</td>
<td>500</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>500</td>
<td>1000</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>7.5</td>
<td>500</td>
<td>1500</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 8-2 Each group consisted of 20 plates. The total amount of plasmid and jet PEI required for different N/P ratio is summarized in the table. Transfection time [incubation] was 4 hours.

Results: In Group I. 4 hour transfection with N/P: 2.5 resulted in poor transfection, while N/P: 5 had significantly better transfection. The highest transfection rate seemed to be present in cells exposed to N/P: 7.5. In these series all cells in each group survived the transfection time and resulted in > 90 % confluence 48 hours after transfection [Figure 8-4].
Figure 8-4 Fluorescent picture showing minimal transfection in Group I. B: Light microscopic picture of 293T cells after 48 hours in Group II. Cell confluence was more than 80-90%. C: Fluorescent picture of the same cell population in Group II. Transfection was high in this group. D: Light microscopic picture of 293T cells after 48 hours in Group III. Cell confluence was more than 80-90%. E: Fluorescent picture of the same cell population in Group III. Transfection was the highest in this group [all pictures 10x magnification].

8.3.8.3 Experiment III. – Effect of 2, 4 and 6h exposure time on transfection rate

Experimental set up: In this experiment we wished to look at transfection efficiency in cells exposed to the transfection mix for 2, 4, and 6 hours with three different N/P ratios, N/P: 2.5 [Group I], N/P: 5 [Group II], N/P: 7.5 [Group III]. Each experimental group had three subgroups depending on exposure time. Each subgroup included 5 x 150 mm plates [total of 45 plates]. After 48 hours, cells were examined with confocal fluorescence microscopy [Table 8-3].

<table>
<thead>
<tr>
<th>Group</th>
<th>N/P ratio</th>
<th>DNA (µg)/5 plates</th>
<th>jetPEI (µl)/5 plates</th>
<th>Transfection time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>2.5</td>
<td>125</td>
<td>125</td>
<td>2,4,6</td>
</tr>
<tr>
<td>II.</td>
<td>5</td>
<td>125</td>
<td>250</td>
<td>2,4,6</td>
</tr>
<tr>
<td>III.</td>
<td>7.5</td>
<td>125</td>
<td>375</td>
<td>2,4,6</td>
</tr>
</tbody>
</table>

Table 8-3 Each group consisted of 5 plates [total of 45 plates]. The total amount of plasmid and jet PEI required for different N/P ratio is summarized in the table. Transfection time [incubation] were 2, 4, 6 hours.
Results: Transfection was insufficient with 2h incubation time in each group. N/P ratio of 2.5 did not result in acceptable transfection. There was no significant difference in transfection when the cells were exposed to 4 or 6 hour incubation time in the N/P: 5 and N/P: 7.5 groups.

8.3.8.4 Summary of experiments to optimize transfection protocol

N/P ratio of 2.5 and 2 hours incubation resulted in poor transfection. There was no significant increase in transfection efficiency when time was increased from 4 to 6 hours. Increasing N/P ratio from 5 to 7.5 was associated minimal increase in transfection. JetPEI exposure at a concentration of N/P: 5 and N/P: 7.5 for 48 hours seemed to be cytotoxic. Based on these observations, we used a transfection time of 4 hours and an N/P ratio of 5 in further experiments.

8.3.9 HARVESTING VIRUS CONTAINING 293-T CELLS

Twenty-four hours after transfection, plates were checked under fluorescent microscope. Transfection was considered acceptable if approximately 50% fluorescence was achieved. If transfection resulted in less than 50%, plates were discarded and the experiment set up again. Method is described in more details in Chapter 3, Section 3.6.3.8.

8.3.10 PURIFICATION OF CELL LYSATE BY IODIXANOL GRADIENT ULTRACENTRIFUGATION

Most of the variation in transduction capacity of rAAV depends on the bulk purification method of the crude lysate which may result in highly contaminant viral preparations or reduced viral vector infectivity [281, 346]. These have been demonstrated systemically by Zolotuknin et al [281], comparing the traditional cesium chloride [CsCl] gradient with iodixanol non-ionic gradient centrifugation developed by the same group. One of the main impediments to achieve pure viral preparation is the aggregation of cell proteins from the crude cell lysate to the viral particles rendering the complex biochemically very heterogeneous. This can interfere with the ionic gradients [such as CsCl] and ion exchange chromatography resulting in highly contaminated viral preparation as well as lower yields. The presence of proteins in the final stock may trigger immune response and may influence the experimental
outcome. Furthermore, conventional purification methods also have been associated with high rate of ineffective viral particles, described by the particle-to-infectivity ratio [total virus particle to the number of infective virus particle determined by plaque assay]. A ratio of 100:1 for example means, that as much as 99% of the vector particles are non-infective. The quality of purification can be illustrated by the fact that the application of an iodixanol gradient with subsequent heparin affinity chromatography results in around 26 particle-to-infectivity ratio while ammonium sulphate fractionation [a step prior to CsCl gradient to reduce the amount of protein-viral aggregate] and sequential CsCl centrifugation has a ratio between 241-1600. In addition, conventional purification takes up 2 weeks often associated with substantially lower viral yield. In contrast with this, iodixanol gradient and subsequent heparin affinity chromatography can be performed within one day with a recovery of 70-80% of the initial infectious viral units [281].

8.3.10.1 Steps of purification using iodixanol gradient

Iodixanol is an X-ray contrast compound used in clinical practice. It is non toxic, non ionic and it can be prepared isosmotic at various densities. These properties provide better conditions for downstream purification steps.

Four individual iodixanol gradients were prepared as outlined in Table 8-4. Zolotuknin et al have demonstrated that various rAAV serotypes have a banding density of 1.266 g/ml which is equivalent to 50% iodixanol solution (w/v). The application of iodixanol gradient slightly less than this, a 40 % gradient [density = 1.23 g/ml] followed by a 60 % of layer provides a capture surface for the rAAV/protein complexes at the 40%-60% interface [281]. In order to reduce the amount of rAAV/protein aggregates in the crude cell lysate, we added 5M NaCl into the 15% iodixanol layer. This destabilized the ionic interaction between macromolecules and prevented the continuous spread of rAAV in the Iodixanol gradient. In the rest of the layers [i.e. in the 25, 40, 60%] we omitted the salt component to make sure that the final viral lysate is isosmotic and suitable for chromatography without any further treatment [such as dialysis]. In order to visualise each individual layer, we added 200 µl of phenol red into the 25% and the 60% Iodixanol density steps [Table 8-4].
8.3.10.2 Preparation of the density gradient

(1) A sterile glass Pasteur pipette was placed into the Optiseal tube and the vector containing cell lysate followed by the iodixanol fractions applied. The solutions were added slowly starting with the lowest density. The increasing concentration of iodixanol fractions displaces the supernatant from below [Figure 8-5].

(2) Total volume of 30 ml was loaded into the Optiseal tubes including 15 ml cell lysate [Figure 8-5].

<table>
<thead>
<tr>
<th>Iodaxinol (%)</th>
<th>Iodaxinol</th>
<th>5 M NaCl</th>
<th>5x PBS-MK buffer</th>
<th>dH₂O</th>
<th>Phenol red</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>12.5 ml</td>
<td>10 ml</td>
<td>10 ml</td>
<td>17.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>25%</td>
<td>20.8 ml</td>
<td>-</td>
<td>10 ml</td>
<td>19.2 ml</td>
<td>200 µl</td>
</tr>
<tr>
<td>40%</td>
<td>33.3 ml</td>
<td>-</td>
<td>10 ml</td>
<td>6.7 ml</td>
<td>-</td>
</tr>
<tr>
<td>60%</td>
<td>50 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

Table 8-4 The table summarises the components of each iodixanol fraction. PBS-MK: phosphate buffer saline - MgK; dH₂O: distilled water.

Figure 8-5 Schematic picture of the Optiseal tube containing the virus lysate and the iodixanol fractions before centrifugation and after centrifugation. The virus containing sample gradually moves down in the gradient and accumulates at the 40-60% interface. The table shows the volume fractions of the different layers before centrifugation.

Based on the Zolotuknin et al experiments, we used one Optiseal tube [Beckman #361625] to purify rAAV cell lysate from 10 [15 mm] plates [281]. According to their observations, 15 plates were associated with significant increase in protein contamination and reduced efficacy of purification. In contrast with this, 10 plates per
gradient resulted repeatedly in $10^{14}$ virus particles at the end of the purification and $10^{12}$ of these were infectious and could be used for transduction.

(3) The loaded Optiseal tubes then were centrifuged at 69,000g for 1 hour at 18°C using a Beckman Ultracentrifuge 70Ti type rotor. Beckman Coulter Ultracentrifuge, Optima LE-80K is rated for 80 000rpm generating a maximum g-force of 548,000g. Type 70 Ti, rated for 70 000 rpm, is a fixed angle rotor designed to centrifuge at a 23-degree angle to the axis of rotation. The rotor develops centrifugal forces sufficient for applications, such as pelleting of small particles, purification of viruses and subcellular organelles.

(4) Following ultracentrifugation, the 40% fraction was removed using first a 21g needle to make a puncture site on the plastic tube, then another 21g needle was placed through the same hole to aspirate the virus containing fraction [we used two needles since the first needle usually was occluded with plastic during puncture]. The bevel of the needle was facing upwards right at the top of the 60% layer and 3 ml 40% fraction was aspirated slowly and continuously to avoid any turbulence. The 3 ml fractions were collected from each Optiseal tube and stored at 4°C until ion exchange chromatography was performed.

8.3.11 ION EXCHANGE COLUMN CHROMATOGRAPHY

Ion exchange chromatography has been used more than 50 years to separate molecules based on their differences in their net ionic charge, charge density, and surface charge distribution. This method is highly selective and able to separate molecules with minor differences in their charge properties rendering the technique suitable for both micro and macro scale rAAV purification. The main principle of the technique is that the net surface charge of a molecule [in this case the protein capsid of rAAV] changes depending on the environmental pH [amino acids are either weak acids or bases]. Changes in the surface charge can be described by the titration curve, which is unique for each protein and reflects how the overall net charge of the protein changes according to the pH of the surroundings. The separation of molecules is achieved by the reversible interaction between the charged molecule and an oppositely charged chromatography surface. The aim is to reversibly bind the molecule
of interest to the chromatography medium and subsequently remove them [now without the presence of other molecules] by applying a stepwise gradient wash with increasing ionic strength, most commonly using NaCl [346]. The steps of ion exchange column chromatography are described in Chapter 3, Section 3.6.3.10.

8.3.12 CONCENTRATION AND DESALTING OF PURIFIED AAV6 VECTOR

The aim of this step to remove the high salt containing buffer fraction from the eluent [Buffer B contains 500 mM NaCl] and concentrate the viral stock to a final volume between 200-500 µl using serial centrifugation steps. We used Amicon® Ultra-15 centrifugal filter device [Millipore UFC910008]. The applied method is described in Chapter 3, Section 3.6.3.11.

8.3.13 REAL TIME QUANTITATIVE PCR-BASED TITRATION OF AAV6 STOCKS

To determine the titre of viral preparation we used the Real Time-PCR method. This step included the determination of standard curves using a stock of plasmid of known concentration [pAAV-MCS-catalase]. Subsequently, our viral stock was compared to the standard curve and the concentration of the viral titre determined.

8.3.13.1 Calculation of the standard curve using pAAV-MCS-catalase plasmid with CMV promoter

(1) Mass of one plasmid DNA (ng)

Length of the plasmid pAAV-MCS-catalase: 6283 base pair (bp)

Average weight of 1 bp in Dalton (Da): 650 Da

Weight of the plasmid pAAV-MCS-catalase: 4083950 Da

Weight of 1 Da in g (conversion factor): $1.66054 \times 10^{-24} \text{ g}$

Weight of the plasmid pAAV-MCS-catalase: $6.78156 \times 10^{-18} \text{ g}$

**Weight of the plasmid pAAV-MCS-catalase (ng):** $6.78156 \times 10^8 \text{ ng}$

(2) Calculation of pAAV-MCS-catalase plasmid content in 1 µl stock

Concentration of the pAAV-MCS-catalase stock: 629.4 ng/µl
Weight of one plasmid pAAV-MCS-catalase: \(6.78156 \times 10^{-8}\) ng

Volume of one plasmid in the known stock: \(9.79428 \times 10^{-11}\) µl

1 µl of stock contained \(\sim 10^{10}\) plasmid pAAV-MCS-catalase.

(3) Standard curve preparation

0.97 µl of pAAV-MCS-catalase was diluted with dH2O to a total volume of 100 µl. This meant that 100 µl contained \(10^{10}\) plasmids. We used this volume to prepare the serial 10x dilutions for the standard curve [Table 8-5].

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Total volume 100 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^8)</td>
<td>1 µl of stock + 99 µl dH2O</td>
</tr>
<tr>
<td>(10^7)</td>
<td>10 µl (10^8)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^6)</td>
<td>10 µl (10^7)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^5)</td>
<td>10 µl (10^6)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^4)</td>
<td>10 µl (10^5)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^3)</td>
<td>10 µl (10^4)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^2)</td>
<td>10 µl (10^3)+90 µl dH2O</td>
</tr>
<tr>
<td>(10^1)</td>
<td>10 µl (10^2)+90 µl dH2O</td>
</tr>
<tr>
<td>Blank</td>
<td>100 µl dH2O</td>
</tr>
</tbody>
</table>

Table 8-5 Table describes the serial dilution of pAAV-MCS-catalase. This served as a standard curve to determine the actual viral titre of the purified stock.

8.3.13.2 Preparation of the rAAV viral sample for real time-PCR

Free plasmids outside the viral capsule which may interfere with the final virus titre were removed using DNAse [Ambion 10x, #083P24A]. These steps are described in Chapter 3, Section 3.6.3.12.
8.3.13.3 Real-time-PCR

We used a total volume 10 µl for each sample as a reaction mix for RT-PCR. This consisted of 1 µl of sample and 9 µl of master mix components [Table 8-6]. Primer sequences were ordered from MWG-Biotech GmbH.

(1) CMV forward sequence: TGG AAA TCC CCG TGA GTCAA

(2) CMV reverse sequence: CAT GGT GAT GCG TTT TGG.

| Reaction mix |  
|--------------|--------------------------------------------------|
| Sample       | 1 µl                                             |
| Fast SYBR® Green Master Mix: | 5 µl                                              |
| Forward primer: | 0.01 µl [0.1µM]                                 |
| Reverse primer: | 0.01 µl [0.1µM]                                 |
| dH2O:          | 3.98 µl                                          |
| **Total volume:** | **10 µl**                                      |

*Table 8-6* Components of the Reaction mix for Real-time-PCR.

All standard samples were duplicates, and the viral samples were triplicates. The total volume of master mix was calculated as it corresponded to the number of the samples.

The cycle set up in the StepOne Plus Real Time-PCR System [Applied Biosystem] was as follows:

(1) 95°C for 2 minutes.

(2) 95 °C for 15 seconds followed by 60°C for 30 seconds [annealing and extension]. This sequence was repeated 40 times.

A standard curve was constructed using the pAAV-MCS-catalase dilutions and used to calculate the rAAV sample CMV concentration which represented the actual virus titre.

8.3.13.4 Calculation of rAAV titre after Real-time PCR

1. *Amplification plot*: Real time PCR results are visualized in an amplification plot to determine $C_T$ value. In the graph, fluorescence is represented on the Y axis [$Rn$],
whereas the number of PCR cycles is plotted in the X axis. The cycle number when the fluorescence of the majority of samples is just entering exponential increase [called threshold], is known as the \( C_T \) (cross threshold) value. The \( C_T \) value is indicative of the amount of target DNA in the starting sample. Higher \( C_T \) means that the DNA product has to go through more PCR cycle in order to reach the threshold indicating that there is less DNA in the original sample [Figure 8-6].

![Amplification Plot](image)

**Figure 8-6** Representative graph from our work. Amplification plot.

2. *Melt curve:* The curve tests the specificity of the PCR product. Once the PCR cycle is terminated, the samples are heated from 50 °C to 95 °C by 0.5 °C and fluorescence change is measured at each temperature. As an effect of the increasing temperature, the double stranded DNA products become separated [“melting”] and generate a change in fluorescence. If only one gene product, in this case the CMV promoter of the gene of interest, was multiplied than the melting curve has one distinguished peak. Multiple peaks reflect sample impurity [Figure 8-7].
3. Standard curve: The curve describes the individual $C_T$ values of the samples of known concentration [pAAV-CMV-catalase 10 x serial dilution]. $C_T$ values from the measured samples were matched to the standard curve and the final titre was determined [Figure 8-8].
Figure 8-8 A representative graph from our work. A standard curve.
8.4 DISCUSSION

8.4.1 BACKGROUND

Historically, AAV protocols consisted of a three plasmid system where co-transfection of cells was carried out using AAV plasmid [containing the transgene in question], helper/packaging plasmid [wild type AAV containing rep/cap genes] and low concentration of adenovirus [containing helper genes for replication] [225, 279]. The main disadvantage of this system that cells may be affected by the adenovirus itself which can be pathogenic and lead to cell lysis. Also, the prevalence of neutralizing antibodies against wild type AAVs are very common in humans, therefore it is inevitable that the accidentally produced wild type AAVs may trigger immune response and modify the expected effect of the delivered transgene. In addition, the application of complex transfection system with multiple plasmids may increase the variability in vector yields and may impede the scale-up of the protocol. To overcome the previously mentioned drawbacks of AAV production, Grimm et al have developed a two plasmid system, termed the “Helper-virus free system”, and constructed a helper plasmid containing the Rep/Cap genes with adenoviral genes [E4, E2a, VA] [279]. In this case, co-transfection was 10 fold more efficient compared to three plasmid system, particularly when AAV2, AAV3 and AAV6 Rap/Cap serotypes were used. The helper construct also contained a DNA sequence for fluorescent protein to detect transfection and visualize transfection efficiency by fluorescent microscope. Similarly to Grimm et al’s vector system, we utilised a pAAV-IκBα-SR plasmid containing GFP DNA sequences, and a second helper plasmid containing the Rep/Cap genes in association with the necessary adenoviral helper genes [Figure 8-1 C-E].

8.4.2 RESULTS AND LIMITATIONS

In order to generate the required amount of rAAV vector containing IκBα-SR transgene or null vector for in vivo experiments, we used a protocol which has been well described in the literature and has been repeatedly used by our group to realize gene delivery to rodents [233, 281, 346]. The AAV plasmid containing the transgene in question also contained a DNA segment for green fluorescent protein [GFP] in order to monitor transfection efficacy in 293T cells during the vector generation process. We
optimized cell transfection, by using 4 hour incubation time with an N/P ratio 5. This resulted in a more than 70-80% transfection rate after 48 hours [Section 8.3.8].

Zolotuknin et al have described that recombinant AAV purification process using iodixanol gradient centrifugation and heparin affinity chromatography resulted repeatedly in a final virus titre of $10^{14}$ viral particle/$\mu$l with an effective infectious viral stock of $10^{12}$ particle/$\mu$l [281]. We used ion exchange chromatography instead of heparin affinity chromatography in the purification process since this method has proven reliably provide a virus titre around $10^{13}$ particle/$\mu$l in our laboratory.

In contrast with this, our final AAV6-IκBα-SR titre varied between $10^7$-$10^9$ particles/$\mu$l. This was substantially less than reported in the literature and by our group [233, 281]. The reason for this may be explained, at least in part, by the following factors:

(1) The NF-κB pathway plays an essential role in regulating cell proliferation [347-349]. However, persistent high NF-κB activity can lead to malignant cell transformation and immortalize human cells [349]. In addition, the presence of inactivating mutations to reduce IκBα activity also have been identified as carcinogenic factor. Mounting preclinical data have shown that inhibition of the NF-κB pathway via gene transfer can reduce tumour cell proliferation, facilitate apoptosis and can be used as an adjunctive to increase the sensitivity of cancer cells to radiotherapy or chemotherapy [348]. We used human embryonic kidney cells [293T] for our transfection. These cells are originally generated by exposing 293T cells to adenovirus 5 DNA fragments and are immortal and express features similar to cancer cells [350]. As it follows, it is possible that transfection of 293T with IκBα-SR transgene may have inhibited endogenous NF-κB activity, similarly to the observations in other cancer cells, and impeded in some degree the synthesis of recombinant viruses. This assumption can be partially supported by the fact that rAAV6-Null vector [not carrying the IκBα-SR transgene] titres were repeatedly higher than the viral titres for rAAV- IκBα-SR.

(2) We used green fluorescent protein to assess transfection efficiency. However, in the gene expression cassette the IκBα-SR transgene and GFP sequences were separated by an ITRS segment and their transcription resulted in two individual
proteins inside the cells [IκBα-SR and GFP] instead of a large IκBα-SR-GFP conjugate protein. This arrangement may have introduced bias in the determination of transfection efficiency, since cells may have had only GFP, GFP and transgene, transgene only, or not transfected. Clear distinction among these cells was not possible with our technique.

(3) One of the disadvantages of rAAVs is their low packaging capacity, limiting transgene expression to a size of less than 4.7 kbp [220]. The entire IκBα-SR plasmid construct is 4.5 kbp, therefore it is possible that certain percentage of the viral construct was defective and did not contain the transgene, resulting in lower yield as we expected. Of note, our lab successfully constructed high titre of rAAV6 vectors containing EC-SOD transgene, however EC-SOD is much smaller molecule [723 bp] than IκBα-SR [4.5 kbp] [233]. Furthermore, the presence of EC-SOD does not interfere with cell proliferation [the protein is secreted to the extracellular space] providing better transfection conditions than transfection of an NF-κB inhibitor.

(4) Purification steps can affect final vector yield and the quality of the viral preparation. In addition, it has been shown that total virus titre after virus purification may not be equivalent with the actual infectious titre [i.e. the number of effective recombinant virus which can transduce living cells]. Zolotukhin et al demonstrated that the above described protocol results in 70-80% virus recovery and $10^{12}/\mu l$ infective virus titre from 40 x 150mm plates. In contrast, the majority of our purification process yielded to $10^9/\mu l$ virus titre indicating that the actual infective viral construct may have been less than $10^5/\mu l$.

8.5 CONCLUSION

(1) Our final titres varied between $10^7$ - $10^9$ viral particle/$\mu l$ rAAV6-IκBα-SR vector and $10^9$ viral particle/$\mu l$ rAAV6-Null vector. This was sufficient to carry out a three group in vivo experiment including 10 animals in each group [total of 30 animals].

(2) The lower rAAV yield during vector generation may have been related to the effect of the transgene on cell function, lower packaging capacity of AAV vectors and to the purification process.
CHAPTER 9
CHAPTER 9: DETERMINATION OF THE EFFECT OF OVEREXPRESSION OF
THE NF-κB INHIBITOR IκB-α IN VENTILATION INDUCED LUNG INJURY

9.1 ABSTRACT

Background: The NF-κB pathway is one of the main regulators mediating the
inflammatory response during mechanical stretch induced lung injury. Inhibition or
attenuation of N-κB activity therefore may be considered as a possible therapeutic
target in ALI/ARDS to reduce tissue injury. It has been demonstrated that
intrapulmonary delivery of transgenes inhibiting IκB-α degradation reduces ischaemia-
reperfusion, endotoxin and acute pneumonia induced lung injury. Therefore, we
hypothesised that overexpression of IκB-α-super-repressor [IκBα-SR] transgene, a
potent inhibitor of the NF-κB pathway, could reduce the severity of lung injury in a
rodent model of VILI.

Methods: Adult, male Sprague-Dawley rats were utilized. Series I.: 4 rats randomly
received surfactant alone [Vehicle, n=2] or 1x10¹⁰ AAV6 vector particles containing
IκBα-SR transgene [AAV6- IκBα-SR, n=2] via intratracheal instillation. After 96 hours,
animals were sacrificed and lungs were removed to measure transgene delivery in lung
epithelial cells using flow cytometry. Series II.: Animals were anaesthetised and
randomly allocated to undergo intratracheal instillation of surfactant alone [Vehicle,
n=10], 1x10¹⁰ particle of AAV6 without transgene [AAV6-Null, n=10] or 1x10¹⁰ AAV6-
IκBα-SR [n=9]. After 96 hours animals were exposed to 4 hours of injurious ventilation
[PIP: 22.5 cmH₂O, PEEP: 0 cmH₂O, RR: 20 breaths/min, FiO₂: 0.3]. Survival, lung injury
and inflammatory markers, transgene delivery and histological injury were assessed.

Results: in Series I., the delivery of 1x10¹⁰ AAV6-IκBα-SR delivery resulted in ~21.4%
transfection rate in lung epithelial cells. In Series II., AAV6-IκBα-SR pre-treatment
improved oxygenation and lung permeability, as evidenced by better oxygenation in
animals exposed to FiO₂: 1.0 at the end of the experiment, and reduced BAL protein
count compared to AAV6-Null and Vehicle groups. However, other lung injury markers
were not different among the groups. AAV6-IκBα-SR delivery resulted in a moderate,
but significant IκBα-SR transgene expression in lung tissue. Importantly, given
concerns regarding immunogenicity of viral vectors, AAV6 viral vector administration did not worsen inflammatory response during VILI.

**Conclusion:** Our results do not clearly confirm the hypothesis that inhibition of pulmonary NF-κB activity reduces ventilation induced lung injury. This may be due, in part, to suboptimal expression of the IκBα-SR transgene, notwithstanding the demonstration that AAV6-IκBα-SR tracheal instillation did result in successful gene delivery in lung epithelial cells. Reassuringly, AAV6 viral vector delivery to the lungs appeared to be safe in VILI.
9.2 INTRODUCTION

Despite ongoing intensive research, there are no specific therapies for ALI/ARDS. Up to date the cornerstones of ARDS management are mainly supportive and limited to protective mechanical ventilation [5] and restrictive intravenous fluid management [58]. Rescue therapies, such as prone positioning [351, 352], NO administration [60], HFOV [78] and ECMO [80] may improve oxygenation in severe ARDS. However, these therapies have not been associated with mortality benefit. Pharmacological therapies that have been tested without success include pulmonary vasodilators [61], antioxidants [62, 63], and surfactant [64]. Similarly, corticosteroids [65], immunomodulatory agents such as granulocyte- macrophage colony stimulating factor[66] failed to improve outcome in ALI/ARDS. Consequently, novel, “non conventional” therapeutic approaches, such as gene therapy, could open up new opportunities in the management of ALI.

9.2.1 ADVANTAGES OF GENE THERAPY IN ALI/ARDS

The essence of gene therapy is to insert genes or small nucleic acid sequences into cells to replace the function of a defective gene, or to alter the production of a specific gene product. This powerful methodological approach can potentially contribute in two main areas of research in ALI/ARDS. (1) Gene delivery can be a therapeutic option in ALI/ARDS for several reasons. The disease process has a transient nature, and therefore short term gene expression approaches may be sufficient to produce a therapeutic effect. Both the distal epithelium, via tracheal administration, and the pulmonary endothelium from the circulatory route, are easily accessible for gene transfer. This may provide more specific, cell targeted and disease specific gene therapy. In addition, there is a possibility to modify or treat separately the initial injury and the reparative phase of ARDS. (2) Gene therapy can also expand our understanding on the pathophysiology of ALI/ARDS by providing a possibility to study signal transduction pathways in live animals. Moreover, proof of concept studies involving intracellular mechanisms can be modelled using “wild-type” instead of genetically manipulated [transgenic] animals making the in vivo model more realistic and relevant.
9.2.2 CHALLENGES OF GENE THERAPY IN ALI/ARDS

Effective gene transfer to the lung represents a unique challenge in gene therapy [217, 218]. One of the requisites of successful gene delivery is the application of vector systems that are able to carry a large amount of genetic material into the cells. However, many hurdles need to be overcome to realize effective gene delivery. These includes: (1) various pulmonary defence mechanisms against inhaled particles [217, 219]; (2) the inflammatory state of the injured lung that can limit gene transfer secondary to loss of alveolar epithelium, pulmonary oedema formation, and increased bronchial plugging by mucus and cell debris [218]; (3) limitations of vector systems, such as lack of efficiency of gene transfer and gene expression [specially in non viral vector systems], increased immunogenicity [viral vector systems] and limited transgene packaging capacity.

9.2.3 MODULATION OF NF-κB PATHWAY VIA VIRAL VECTORS IN ALI/ARDS

AAVs are single stranded DNA Parvoviruses that require helper viruses, such as herpes simplex or adenovirus to replicate [220]. The main advantage using AAV as a delivery system relates to its lack of pathogenicity in humans. This virus family is less immunogenic compared to other viruses and confers long term stable gene expression both in dividing and non dividing cells. Most importantly, different AAV serotypes have specific tissue tropism which can be used to achieve tissue/cell targeted gene delivery. AAV6 serotype has been demonstrated to have good transduction efficiency for healthy and diseased lung in various in vivo experimental models [221, 233, 234, 282, 283, 344] [See Chapter 8, Section 8.2.3.3-8.2.3.4].

Nuclear factor-κB plays an essential role in the evolution as well as the resolution phase of inflammation in ALI/ARDS [216]. Selective transduction of airway epithelial cells in vivo by adenoviral vectors [Ad], containing either IKK-α or IKK-β transgenes, results in NF-κB activation, induction of mRNA expression of several NF-κB dependent cytokines, increased level of BAL cytokines and neutrophil counts and significant neutrophil infiltration in lung histology [209]. Co-administration of Ad expressing a dominant inhibitor of NF-κB [IκB-αDN] prevents lung inflammation in these animals [209]. Although NF-κB seems to drive inflammation and is responsible for tissue injury
in response to various noxious stimuli, such as ischaemia-reperfusion [213], stretch [96], and sepsis induced lung injury [210, 212, 353], increased activity of the NF-κB pathway is also essential for bacterial clearance, host defence [137, 214, 215] and may play a key role in lung repair [134]. Consequently, blocking the NF-κB pathway can potentially result in both beneficial and harmful effects depending on the inciting stimulus, cell type involved in the inflammation, and on the phase of the injury process.

The facts that currently no specific therapy exist to treat ALI/ARDS and that the main supportive measure in this disease [i.e. mechanical ventilation] may convey significant harm to the lungs, support the idea that inhibition of NF-κB activation in the acute phase of VILI via gene transfer may be used as a therapeutic adjunctive to protect the lungs during injurious mechanical ventilation. Therefore, we hypothesised that pulmonary over-expression of IκBα-SR transgene using AAV6 vector delivery system would attenuate high mechanical stretch induced lung injury in an in vivo rodent model.
9.3 METHODS

Specific-pathogen-free adult male Sprague Dawley rats were used. All work was approved by the National University of Ireland, Galway Animal Care Research Ethics Committee and conducted under license from the Department of Health, Ireland.

9.3.1 VECTOR INSTILLATION

Animals were anesthetized by inhalational induction with Isoflurane and intraperitoneal injection of 40 mg/kg ketamine. After confirmation of depth of anaesthesia, laryngoscopy was performed and the trachea intubated with a size 14 intravenous catheter. Animals were randomized to intratracheal instillation of 250 μl of a surfactant/PBS mixture containing: (1) surfactant alone [Vehicle, n=10]; (2) 1 x 10^{10} AAV IκBα-SR [AAV6-IκBα-SR, n=9]; (3) 1 x 10^{10} AAV-Null [AAV6-Null, n=10]. After instillation, the animals were extubated and placed in an individually ventilated recovery cage for 96h to allow maximal transgene expression in the lungs [See Chapter 3, Section 3.4].

9.3.2 SERIES I: DETREMINATION OF IκBα-SR TRANSDUCTION RATE IN LUNG EPITHELIAL CELLS 96 HOURS FOLLOWING IN VIVO VECTOR INSTILLATION

We wished to determine whether intratracheal instillation of AAV6-IκBα-SR transgene results in stable and reproducible transgene expression in uninjured rat lungs. 2-2 animals underwent surfactant alone [Vehicle] and 1x10^{10} AAV6 IκBα-SR intratracheal instillation. After 96 hours animals were exsanguinuated and placed in an individually ventilated recovery cage for 96h to allow maximal transgene expression in the lungs [See Chapter 3, Section 3.4].

9.3.2.1 Preparation of single cell suspension from lung tissue

Immediately after exsanguination lung tissue was removed and finely chopped from randomly selected lung areas. Lung tissue was then placed in a 15 ml tube and 3 ml of Collagenase Type I was added and digested at 37°C degrees for 2 hours. The tissue preparation was vortexed every 20 minutes. After 2 hours, the thick lung homogenate was filtered to remove debris and the cell suspension was collected in a 50 ml tube. Subsequently 10-20 ml DMEM was used to stop digestion and to wash through the filter and the cell suspension was centrifuged at 1500 rpm for 10 minutes. The
supernatant was discarded and the cell pellet resuspended in 3 ml of red cell lysis buffer [ACK lysis buffer: 150mM NH₄Cl, 10mM KHCO₃ 0.1mM Na₂EDTA] for 2 minutes. After that, 10 ml DMEM was added to dilute red cell lysis buffer and the mix was transferred into a 15 ml tube and centrifuged at 1500 rpm for 10 minutes. Debris free cells resulted in a white cell pellet and were subsequently resuspended in 10 ml FACS buffer [1% BSA, 0.1% NaN₃ in PBS, filtered]. The suspension was allowed to stand on ice for 10 minutes. From the top 7 ml cell suspension was filtered again to remove any remaining cell clumps and the sample was stored on ice prior to FACS analysis.

9.3.2.2 Antibody staining of lung epithelial cells from single cell suspension

Two ml of cell suspension [c. 5x10⁶ cells/ml] were placed into a 15 ml tube and 40 µl 1:20 concentration of epithelial specific antibody [Anti-pancytokeratin-11 antibody, #:ab52460; Cambridge, UK] was used to identify lung epithelial cells. The mix was kept on ice and in the dark for 20 minutes and washed with FACS buffer. Staining was performed with ice cold reagents and solutions, since low temperature and presence of NaN₃ [sodium azide] prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity. After 20 minutes the suspension was centrifuged at 1500 rpm for 10 minutes and the cell pellet resuspended in 1 ml FACS buffer.

9.3.2.3 Flow cytometry and analysis of lung epithelial cells

Morphological and numerical analysis of single cell suspension was performed by plotting LASER beam side scatter [SSC] values against forward scatter [FSC] values. Subsequently, cells tagged with phycoerythrin-conjugated epithelial specific antibody [Anti-pancytokeratin-11 antibody, #:ab52460; Cambridge, UK] were identified. This cell subpopulation represented epithelial cells from the lung homogenate. Fluorescein isothiocyanate [FITC] excitation [488nm] was used to calculate the percentage of lung epithelial cells that were transduced with IκBα-SR-GFP.
9.3.3 SERIES II: DETERMINATION OF POTENTIAL FOR IκBα-SR OVEREXPRESSION TO ATTENUATE VILI

9.3.3.1 Experimental protocol and animal surgery

Ninety-six hours following intra-tracheal instillation of the vector, when transgene expression was determined to be optimal in prior experiments and suggested in previous publications [209, 233], animals were re-anesthetized. Following induction of anesthesia, a tracheostomy was inserted and arterial and venous access secured, animals underwent mechanical ventilation with FiO₂: 0.3; peak inspiratory pressure 22.5 cmH₂O; respiratory rate 20/min; PEEP 0 cmH₂O for a period of 4 hours. These ventilation settings were determined to produce a significant lung injury after 4 hours in this animal batch in a small pilot series. Maintenance of anaesthesia and animal surgery was carried out the same way as it has been described earlier [Chapter 3, Section 3.3.7]. Exclusion and termination criteria were defined as it has been described in the Methods chapter [Chapter 3, Section 3.3.6]

9.3.4 MEASUREMENT OF PHYSIOLOGICAL VARIABLES, TISSUE SAMPLING AND ASSAYS, STEREOLOGICAL ANALYSIS

Measurement of physiological variables are described in Chapter 3, under Section: 3.5.1, tissue sampling and assays, including measurement of BAL protein, total and neutrophil cell count, cytokine levels are discussed in Chapter 3 under Sections: 3.5.2-3.5.6, histological preparatums and stereological analysis is described in Chapter 3, Sections: 3.5.10-3.5.11.

9.3.5 REAL TIME-PCR TO DETERMINE IκBα-SR-GFP TRANSGENE INDUCTION AND ENDOGENOUS IκB-α GENE INDUCTION AFTER INJURIOUS VENTILATION

We tested IκBα-SR transgene expression in lung tissue independently from endogenous IκBα expression using real-time PCR. Two-hundred mg of lung tissue from each animal’s right lung was used to obtain RNA extracts. The prepared RNA was used to produce cDNA and determine IκBα-SR content. cDNA was synthesized from the RNA using the ImProm-II™ reverse transcription system from Promega® (2800 Woods Hollow Road Madison, WI, USA). Each cDNA sample was subjected to duplicate analysis. Quantitative PCR was performed for human IκBα-SR and rat IκB-α,
normalised against a Lamin A/C control product. We looked for a comparison of fold induction of human IκBα-SR and rat IκB-α amongst the 3 groups of animals [Vehicle, AAV6-transgene, AAV6-Null], to ensure we had successfully delivered AAV6-IκBα-SR construct to our treatment group [See Chapter 3, Section 3.7.2 for more detailed description of RNA extraction, cDNA synthesis and RT-PCR].

9.4 STATISTICAL ANALYSIS

Distribution of data was tested for normality using the Kolmogorov-Smirnov test. Continuous variables are expressed as mean ± standard deviation [SD] for normally distributed data, and as median [interquartile range, IQR] if non-normally distributed. Response variables that were obtained at multiple time points throughout the experiments, such as paO₂, paCO₂, pH, serum lactate, static compliance, MAP were analysed by two-way analysis of variance, with group allocation as a group factor [Null vector, AAV6-IκB-α, AAV6-Null] and time as a repeated measure. There was no evidence against normality and equal variance for these variables. Data obtained at the end of the experiments, such as wet/dry weight ratio, BAL neutrophil counts, cytokine levels, final PaO₂, and DAaO₂, endogenous IκB-α induction in lung tissue were analyzed, using a Student-Newman-Keuls test. Lung histology was analyzed by two-way analysis of variance, with group as the first factor and histologic classification [ airspace, tissue space, extra-acinar tissue ] as the second factor. Post hoc tests were carried out using 1-way ANOVA as appropriate. A two-tailed p <0.05 was considered significant.
9.5 RESULTS

9.5.1 DEMONSTRATION OF TRANSDUCTION OF LUNG TISSUE AFTER 96 HOURS WITH AAV6-\(\text{i}B\alpha{-}\text{SR}\)

Two animals received Vehicle and 2 animals were transduced with \(1 \times 10^{10}\) AAV6-\(\text{i}B\alpha{-}\text{GFP}\) for 96 hours. A PE labelled epithelial specific antibody was used to identify epithelial cells from the lung homogenate and FITC excitation was used to calculate the percentage of cells that were transfected with GFP. Of the epithelial cells stained by the cytokeratin-PE antibody, 21.4 % were positive for GFP, whereas overall 1.4 % of events were positive for GFP in the surfactant group [Figure 9.1].

9.5.2 EFFECT OF AAV6-\(\text{i}B\alpha{-}\text{SR}\) TRANSGENE DELIVERY ON THE SEVERITY OF VILI

9.5.2.1 Baseline characteristics

Twenty-nine animals were entered into this study. One animal in the AAV6-\(\text{i}B\alpha\) group died during vector instillation due to laryngospasm. There were no differences among the groups at baseline with regard to animal weight, MAP, \(\text{PaO}_2\), \(\text{PaCO}_2\), arterial pH, serum bicarbonate and base excess, and static compliance. Serum lactate was higher at baseline in the Vehicle group compared to AAV6-\(\text{i}B\alpha\) and AAV6-Null [\(p=0.023\)] [Table 9-1].

9.5.2.2 Animal survival

There was no difference in mean survival time [Figure 9-2]. Survival rate at 4 hours was 50% in the AAV6-Null group, 67 and 70% in the AAV6-\(\text{i}B\alpha\) and Vehicle groups, respectively [\(p=0.617\)] [Table 9-1].

9.5.2.3 Acid base balance

Arterial pH significantly decreased over time in each group and \(\text{PaCO}_2\) slightly increased during injurious ventilation, however there was no difference between the groups [Table 9-1]. Serum bicarbonate and base excess decreased significantly from baseline in each group over the course of the protocol, but there were no between group differences [Table 9-1]. Serum lactate also increased over time however, there were no difference among the three groups at the end of the experiment [Table 9-1].
9.5.2.4 Assessment of lung Injury

Overexpression of IκBα-SR transgene decreased the severity of VILI. Arterial O2 tension decreased significantly over time in each group and the change was comparable among the three groups [Table 9-1; Figure 9-3]. Arterial O2 tension on FiO2: 1.0 was significantly higher in animals pre-treated with AAV6-ικBα-SR transgene compared to AAV6-Null and Surfactant [Vehicle] groups [p < 0.05] [Figure 9-4]. Consistent with this, alveolar-arterial O2 gradient was significantly lower in the AAV6-ικBα-SR group, while animals treated with AAV6-Null or Surfactant had higher values [332 ± 99 vs. 454 ± 135, 426 ± 135 mmHg, p<0.05] [Figure 9-5]. Lung compliance was comparable at baseline among the groups, indicating that AAV6 vector with or without transgene instillation did not cause derangement in lung function [Figure 9-6]. Lung static compliance was significantly decreased following VILI [Figure 9-6]. However, there were no significant differences among the three groups upon termination of the experiments [Figure 9-7]. AAV-ικBα-SR instillation decreased BAL protein content, indicating reduced alveolar capillary leak, compared to both AAV6-Null gene and Vehicle [P < 0.05] [Figure 9-8]. In contrast, while there was a trend to reduced lung wet:dry weight ratios, these differences were not statistically significant [Figure 9-9].

9.5.2.5 Assessment of lung inflammation

Although BAL neutrophil counts [Figure 9-10], CINC-1, IL-6 and TNF-α levels were slightly lower in the AAV6-ικBα-SR group these differences were not significant among the groups [Figure 9-11; Figure 9-12; Figure 9-13].

9.5.2.6 Assessment of IκB-α transgene expression in lung tissue

In order to differentiate between endogenous IκB-α gene and instilled IκBα-SR transgene expression in lung homogenates we used primers specific to each IκB-α. These primers were substantially different from each other and had different binding location on the cDNA. AAV6-ικBα-SR treatment resulted in significantly higher transgene induction compared to Vehicle and AAV6-Null groups [Figure 9-14]. Injurious ventilation resulted in increased IκBα expression in AAV6-Null and AAV6-ικBα-SR groups compared to Vehicle group [Figure 9-15].
9.5.2.7 Histology

The extent of the histologic injury was relatively moderate and there were no significant structural differences among the three treatment groups [Figure 9-16].
9.6 DISCUSSION

Our aim was to test whether inhibition of IκB-α activation by over-expressing the IκBα-SR gene in lung tissue would reduce mechanical stretch induced lung injury in rats. We examined first the transduction rate of $1 \times 10^{10}$ AAV6-IκBα-SR-GFP delivered to uninjured lung epithelial cells. The proportion of lung epithelial cells [~15%] from the total cell suspension determined by flow cytometry was comparable to other observations from the literature [354-356], which indicated that our epithelial cell fraction was representative of rat lungs. Subgroup analysis showed that ~21.4% of the epithelial cell population was positive for GFP fluorescence as opposed to 1.23% in the vehicle [surfactant alone] group. This data confirms that lung epithelial cells were transduced with IκBα-SR-GFP transgene after 96 hours, however, it does not give information about other cell populations. Importantly, the transduction rate presented in our results was significantly less that it has been reported by others in in vivo mice models [283, 344]. The discrepancy between our observation and others might be due to the fact that in the mice model 10-70 times more AAV6 vector were used [1-7 x $10^{11}$] and transduction rates were assessed after 1 months as opposed to our 96 hours incubation time. However, Sadikot [209, 214], Ishiyama [213], and Hassett et al [233] demonstrated that 48-96 hours incubation time to deliver IKK-α, IκBα-SR and EC-SOD transgenes were sufficient to detect treatment effects in various in vivo ALI models.

We also used RT-PCR to test IκB-α-SR transgene delivery. Using human and rat IκBα specific primers we were able to differentiate between delivered and endogenous IκB-α gene expression and show that AAV6 mediated transgene delivery resulted in 31 fold IκB-α-SR gene expression.

AAV6-IκBα-SR pre-treatment did not affect survival, hemodynamic response or serum lactate levels in response to injurious ventilation compared to Vehicle and AAV6-Null pre-treatment. Arterial oxygenation, acid base balance and static lung compliance and wet:dry lung weight ratio were comparable among the groups. Importantly, arterial $O_2$ tension on $FiO_2$:1.0 was lower, and $AaDO_2$ was higher in animals treated with AAV6-IκBα-SR and this was associated with a significantly less BAL protein content. BAL inflammatory markers were elevated in same degree in all groups after VILI. Histological injury did not differ among the groups.
9.6.1 LIMITATIONS

The above listed results cannot fully support our hypothesis that NF-κB pathway inhibition by pulmonary administration of AAV6-IκBα-SR attenuates ventilation induced lung injury in vivo. However, there are several limitations to our experiments:

(1) We did not carry out a dose response study to define the optimal dose for the vector-transgene construct. The excessive difficulty to produce large amount of AAV6-IκBα-SR viral vector stocks limited the number of animals we could use and prevented the realization of a dose response experimental series. Therefore, the choice of the dose of IκBα-SR transgene was based on literature review [5, 213, 345] and on the results from previous experiments by our group using AAV6-EC-SOD in LPS induced ALI [i.e. 3x 10^{10}] [233].

(2) A 21.4% transduction rate in lung epithelial cells and 21 fold IκBα-SR transgene expression may have not been sufficient to exert protective effect in VILI. Conversely, the severity of lung injury may have been too excessive to detect any beneficial effect in response to NF-κB inhibition.

(3) Intratracheal instillation of AAV vectors may not be the most efficient gene delivery method since it does not provide homogenous spread in the lung tissue. Alternative gene delivery system, such as nebulizers, may circumvent this problem.

(4) The limited transfer capacity of AAV vectors [4.7 kbp] is well described in the literature therefore, packaging of relatively large transgene, such as IκBα-SR [1566 bp], might have been associated with defective vector particles leading to ineffective gene transfer.

Although, administration of IκBα-SR showed limited protection against VILI, we could detect some beneficial effects in lung permeability and oxygenation during injurious ventilation in animals receiving the AAV6-IκBα-SR construct, suggesting that the experimental outcome was mainly related to methodological problems. This was confirmed subsequently by our group administering AAV6-IκBα-SR constructed by VIAPUR [VIAPUR, 6160 Lusk Blvd., Suite C-101, San Diego, CA 92121] to rats and then exposing them to VILI. AAV6-IκBα-SR pre-treatment significantly improved survival and
arterial oxygenation, reduced BAL neutrophil infiltration and histological injury [Hayes M et al, unpublished data]. Tagging AAV6-\(\kappa B\alpha\)-SR with a FLAG epitope [short DNA sequence attached to the transgene construct] instead of using separately expressed GFP, improved transgene detection. Hayes et al demonstrated that AAV6-\(\kappa B\alpha\)-SR-FLAG administration resulted in 100 fold pulmonary transgene expression [unpublished data]. In these studies dose response experiments showed that a \(1 \times 10^{10}\) AAV6-\(\kappa B\alpha\)-SR dose appeared to be as protective as the higher \(5 \times 10^{10}\) dose. In addition, our group also demonstrated in \textit{in vitro} mechanical stretch induced VILI model that transfection of lung epithelial cells [A549] with \(\kappa B\alpha\)-SR transgene significantly reduced NF-\(\kappa B\) activation and IL-8 production [335].

9.6.2 SUMMARY

There are a number of inferences we can drawn from our study: (1) Finding the appropriate vector system to deliver large genes to the lung, such as \(\kappa B\alpha\)-SR is challenging. This highlights the importance of generating better vector systems in terms of efficiency and feasibility; (2) we administered the transgene prior to VILI, to healthy lung. However, in real life many patients present to intensive care with an already established lung injury. The effect of our transgene may have been entirely different in these circumstances, depending on the aetiology of ALI/ARDS [direct vs. indirect], the stage of the disease and the increased extracellular barrier function of the lung due to inflammation; (3) the fact that AAV6-Null vector did not worsen VILI in our experiments and in Hayes et al [unpublished data] study reassures of the safety of AAV6 administration in excessive mechanical stretch induced lung injury; (4) a large body of evidence attests the fact that inhibition of the NF-\(\kappa B\) pathway by pharmacological inhibitors or overexpression of \(\kappa B\alpha\) inhibitors reduces acute inflammation in various ALI models. Our negative results, therefore, do not out rule that inhibition of the NF-\(\kappa B\) pathway may be protective in the context of VILI.
9.7 CONCLUSIONS

1. Intrapulmonary delivery of AAV6-IκBα-SR was localised to the lung epithelial fraction after 96 hours in uninjured animals compared to vehicle.
2. AAV6 vector delivery with or without transgene did not cause lung injury as reflected in normal arterial blood gases and lung compliance at baseline.
3. $1 \times 10^{10}$ AAV6-IκBα-SR transgene instillation conferred limited protection against VILI in this experimental series. This can be best explained by suboptimal gene expression in lung tissue or large variability of gene transfer due to instillation.
4. Lack of increased immune response in the lungs after AAV6-Null viral vector administration reassures the safety of this vector system.
### 9.8 TABLES AND FIGURES

<table>
<thead>
<tr>
<th>Variable</th>
<th>AAV6-IkBα</th>
<th>AAV6-Null</th>
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<tr>
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<td>Animal Weight [g]</td>
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<td>91 ± 26</td>
<td>93 ± 31</td>
<td>79 ± 20</td>
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<td>22.0 ± 1.4</td>
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<td>-3.9 ± 1.9</td>
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**Table 9-1** Summary of survival, and physiological parameters in animals treated with AAV6 vector containing IKBα-SR transgene [AAV6-IkBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant alone [Vehicle] and subsequently exposed to 4 hours of injurious ventilation. Values are expressed as mean ± SD.

a: significantly different from baseline [p<0.01]
b: significantly different from baseline [p<0.05]
c: significantly different from AAV6-\(\kappa B\)-SR and AAV6-Null [\(p<0.05\)]

**Figure 9-1** Summary of flow cytometry analysis of control [Surfactant] and transfected [AAV6-\(\kappa B\)-SR-GFP] lung epithelial cells. SSC (ordinate in panel A/D, abscissa in panel B-C/E-F): side scatter; FSC (abscissa): forward scatter; PE (ordinate): emission events of phycoerythrin conjugated cytokeratin antibody containing cells; FITC (ordinate): emission events of GFP protein containing cells using fluorescein isothiocyanate filter [GFP excitation peak is 488 nm and matches the spectral characteristics of FITC filter]

**A, D panels:** plots represent total lung cell distribution according to their size and structure from control group [A] and transgene group [D]. 44.4% of the sample is lung cell population [epithelial, endothelial, interstitial and immune cells] in the Surfactant group and 41.7% in the AAV-\(\kappa B\)-SR-GFP group.

**D-E:** plots represent the amount of epithelial cells in the Surfactant [D] and in the AAV-\(\kappa B\)-SR-GFP group [E] [i.e. phycoerythrin labelled cytokeratin antibody positive cells]: 15.0 vs. 15.4%, respectively.

**C-F:** plots represent epithelial cells with FITC fluorescent activity. In the Surfactant group 1.23% of the epithelial cells had FITC fluorescence activity [C]. In the AAV-\(\kappa B\)-SR-GFP group [F] 21.4% of epithelial cells had FITC fluorescence activity confirming the presence of transgene-GFP transduction.

**G:** Histogram comparing control [Surfactant] and transfected cells. 21.4% of lung epithelial cells form transduced lung tissue had FITC fluorescence signal.
Figure 9-2 Mean survival time in animals treated with AAV6 vector containing IkBα-SR transgene [AAV6-IkBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to 4 hours of injurious ventilation. Values are expressed as mean ± SD [p=0.783]. There was no difference in mean survival time among the groups.

Figure 9-3 Graph represents arterial O₂ [paO₂] tension over time in animals treated with AAV6-IkBα-SR, AAV6-Null or Surfactant [Vehicle] 96 hours prior to injury. Values are expressed as mean ± SD. *: significantly different from baseline [p<0.05].
Figure 9-4 Final arterial pO₂ tension [paO₂] values on FiO₂:1.0 in animals treated with AAV6 vector containing IκBα-SR transgene [AAV6-IκBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle] 96 hours prior to injury. Values are expressed as mean ± SD. paO₂ was significantly higher in animals treated with IκBα-SR compared to both AAV-Null and Vehicle groups. [*: p < 0.05, Student-Newman-Keuls test post 1-way ANOVA]

Figure 9-5 Alveolar arterial O₂ difference [DAaO₂] on FiO₂: 1.0 after 4 hours injurious ventilation in animals treated with AAV-IκBα-SR, AAV6-Null or Surfactant [Vehicle] 96 hours prior to injury. Animals treated with IκBα-SR had significantly lower DAaO₂ compared to both AAV6-Null and Vehicle groups [*: p < 0.05, Student-Newman-Keuls test post 1-way ANOVA].
Figure 9-6 Baseline and final compliance in animals treated with AAV6-IkBα-SR, AAV6-Null or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values are expressed as mean ± SD. *: significant from baseline [p< 0.05].

Figure 9-7 Graph represents lung elastance upon termination of experimental protocol in animals treated with AAV6-IkBα-SR, AAV6-Null or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values are expressed as mean ± SD. There was no difference between the groups.
Figure 9-8 BAL protein concentration in animals treated with AAV6 vector containing IкBα-SR transgene [AAV6-IкBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. IкBα-SR administration resulted in significantly less BAL protein content compared to AAV6-Null and Vehicle groups [*: p< 0.05, Student-Newman-Keuls test post 1-way ANOVA].

Figure 9-9 Lung wet:dry weight ratio [Wet/Dry ratio] representing lung oedema formation in animals treated with AAV6-IкBα-SR, AAV6-Null or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values are expressed as mean ± SD. There was no difference among the groups.
Figure 9-10 BAL neutrophil counts in animals treated with AAV6 vector containing IκBα-SR transgene [AAV6-IκBα-SR], AAV6 vector without transgene [AAV6-Null] or Vehicle, and subsequently exposed to injurious ventilation. Values expressed as median [IQR]. There was no difference between the groups.

Figure 9-11 BAL CINC-1 values from animals treated with AAV6 vector containing IκBα-SR transgene [AAV6-IκBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values expressed as median [IQR]. There was no difference between the groups.
Figure 9-12 BAL IL-6 values from animals treated with AAV6 vector containing IκBα-SR transgene [AAV6-IκBa-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values expressed as median [IQR]. There was no difference between the groups.

Figure 9-13 BAL TNF-α values from animals treated with AAV6 vector containing IκBα-SR transgene [AAV6-IκBa-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Values expressed as median [IQR]. There was no difference between the groups.
Figure 9-14 \( \text{IkBa-}\alpha-\text{SR} \) transduction resulted in 21 fold transgene expression in lung tissue compared to Vehicle [Surfactant only] and AAV6-Null groups.

Figure 9-15 Endogenous \( \text{IkB-}\alpha \) fold induction in lung tissue at the end of injurious ventilation. There was no significant difference among the groups.
Figure 9-16 Histogram representing stereologic assessment of the extent of histologic injury from animals treated with AAV6 vector containing IKBα-SR transgene [AAV6-IkBα-SR], AAV6 vector without transgene [AAV6-Null] or Surfactant [Vehicle], and subsequently exposed to injurious ventilation. Acinar volume fraction represents all alveolar tissue including epithelium, endothelium, connective tissue and inflammatory cells. Non-acinar fraction means bronchial or vascular space, and airspace describes the gaseous component of the lung. Values expressed as mean ± SD. There was no significant difference between the groups.
CHAPTER 10
CHAPTER 10 DISCUSSION

10.1 ARDS AND VENTILATION INDUCED LUNG INJURY - CURRENT KNOWLEDGE

10.1.1 IMPACT OF ARDS

Acute respiratory distress syndrome constitutes the leading cause of death in critically ill patients. Although some investigators reported that ARDS-associated mortality has decreased over time [357], the estimated mortality rate attributed to ARDS - in spite of many advancements in critical care - remains high, causing approximately 75,000 deaths annually in US alone [7, 8]. This number is comparable to the deaths due to breast cancer or HIV [7]. The most recent retrospective evaluation of data from 4188 patients with ARDS has shown that mortality rates attributed to ARDS varies between 27-45% depending on the severity of the disease [6]. However, when ARDS occurs in the setting of multisystem organ failure, mortality rates may exceed 60%, with significant long term morbidity [7, 10]. Importantly, among 50-70% of survivors who return home, many have persistent physical and neurocognitive sequelae [16, 17, 20, 21] that lead to reduced quality of life and increasing economical burden to individual families and to society as a whole [11].

10.1.2 TRADITIONAL VERSUS PROTECTIVE VENTILATOR STRATEGIES FOR ARDS

Mechanical ventilation has gone through a dramatic evolution over the last sixty years and has saved many human lives. After the Copenhagen polio epidemic in 1952, positive pressure ventilation became one of the most important therapeutic interventions in critical care [358]. Traditional ventilator settings to ventilate patients with ARDS included higher tidal volumes [10-12 ml/kg] with the primary aim of achieving normal or near normal oxygen and carbon dioxide levels in the blood. However investigators increasingly recognized that mechanical ventilation targeting these aims was itself harmful. Insights from pre-clinical studies in combination with observations using new technologies [e.g. computed tomography] elucidated some of the underlying mechanisms of ARDS. These can be summarized as follows: (1) mechanical ventilation can worsen or even cause ALI/ARDS by overdistension and by atelectrauma [50, 53, 54, 101]; (2) mechanical ventilation can activate the cellular and humoral immune system and perpetuate or cause ALI/ARDS. This has been termed as
biotrauma [56, 57, 86]. Translation of this knowledge into clinical practice was the acceptance of low tidal volume ventilation [6 ml/kg] with plateau pressures no greater than 35 cmH₂O and higher arterial CO₂ levels, termed permissive hypercapnia [71, 72]. This was a result of a landmark randomized controlled clinical trial in 2000 – ARMA -, demonstrating a significant reduction in overall mortality in those patients receiving a protective ventilator strategy as opposed to a traditional ventilation strategy [5].

10.1.3 ROLE OF HYPERCAPNIA IN PROTECTIVE VENTILATOR STRATEGIES

Hickling et al suggested first that acceptance of higher arterial blood CO₂ levels in ARDS patients by using low stretch ventilation strategies may be associated with better outcome [48]. Elevated CO₂ level in the presence of low intensity mechanical ventilation subsequently was termed as "permissive hypercapnia" and was incorporated into the management of patients with ARDS.

The protective effects of low tidal volume ventilation in conjunction with permissive hypercapnia were initially thought to be solely due to reductions in lung stretch with much initial experimental [50, 51, 82, 103, 106, 108, 116-118, 268, 271, 317] and clinical [5, 67, 74, 120, 121] research focused on the harm caused by traditional ventilation strategies. In parallel, early experimental work came to light focusing on the physiological effect of hypercapnia on the healthy and injured lung. These studies showed that hypercapnia given into the breathing circuit increased arterial oxygenation, improved V/Q inequalities and lung mechanics [143-147]. The concept that hypercapnia may be beneficial independently from lung protective ventilation led to a proposed paradigm shift considering hypercapnia as “therapeutic” rather than “permissive” entity and suggested that hypercapnia may have an independent immunomodulatory effect in patients with ALI/ARDS [71, 72]. This was subsequently systematically tested in diverse in vivo and ex vivo ALI models. [See Table 1-9 in Chapter 1, Section 1.4.1]. The key observations from these experiments were that HCA attenuated acute inflammation via decreased neutrophil recruitment and function, reduced generation of pro-inflammatory mediators, and reduced oxidative injury and apoptosis. The consequence of this conceptual shift led to the secondary analysis of
the ARMA study whereby Kregenow et al highlighted the possibility that hypercapnia itself might have an independent beneficial effect in ALI/ARDS [69].

10.2 ARDS AND THE NF-κB PATHWAY

10.2.1 IMPORTANCE OF THE NF-κB PATHWAY IN ARDS

ARDS is caused by various direct and indirect injury mechanisms [see Chapter 1, Section 1.1.3.1]. However, it seems that the final manifestation of the pathological symptoms - such as neutrophil sequestration, alveolar-epithelial injury and pulmonary oedema – is shared by the diverse aetiologies of ALI/ARDS [24, 38, 40]. Although these common pathologies can be present in varying degrees and combinations, the assumption that they may be mediated by a common pathway or pathways in ALI, have been suggested by many [27, 216].

10.2.1.1 Role of NF-κB in lung inflammation and injury

The essence of cell activation to stress is increased gene expression and biosynthesis of pro-inflammatory mediators. This is regulated by transcription factors. NF-κB has been considered as one of the key transcription factors coordinating these processes in ALI/ARDS [216]. This concept has been supported a by large body of evidence showing: (1) NF-κB activation in alveolar macrophages is necessary to initiate inflammatory response in ALI and to fight against invading pathogens [91, 135, 136, 359]; (2) NF-κB activation in neutrophils is central to the amplification of the initial inflammatory signal, host defence, and tissue injury [39, 41, 360]; (3) increased NF-κB activity also decreases neutrophil apoptosis, thereby perpetuating the inflammatory response in ALI [39, 216]; (4) activated endothelial cells increase the expression of various adhesion molecules and chemokines via NF-κB activation and facilitate neutrophil and lymphocyte trafficking to the lung [46, 92, 190]; (5) direct injury to epithelial cells activates the NF-κB pathway and can initiate and maintain inflammation in ALI/ARDS [98, 209, 211, 212, 303]; (6) alveolar macrophages isolated from patients with severe ARDS had significantly higher NF-κB activation than control patients without ALI [204, 205]; (7) furthermore, early increase in NF-κB activity in stimulated neutrophils collected from patients with sepsis induced ALI/ARDS was associated with worse clinical outcome, defined as death or less ventilator free days than in control ventilated
patients without ARDS [206]. All these data strongly indicate that NF-κB pathway may be one of the common routes to cell activation in ALI/ARDS. The universal role of NF-κB in inflammation is supported by these studies showing that diverse intracellular processes in many different cell types converge at this pathway.

10.2.1.2 Role of NF-κB in resolution of inflammation and lung repair

The outcome of organ repair and injury resolution after ARDS has significant long term consequences [10, 12, 13]. Interventions, such as mechanical ventilation, and their side effects may promote or delay reparative processes in ALI/ARDS.

Activation of the NF-κB pathway is important in the resolution of acute inflammation [45, 46] and tissue repair [191]. It has been shown that NF-κB has a key role in cell survival and wound repair in intestinal epithelial cells [See Chapter 1, section 1.5.2.3][191, 192]. The role of NF-κB pathway in lung tissue repair has not been investigated directly. Most recently, our group added some insights linking NF-κB activation and wound repair in lung epithelial cells. Although the primary experimental aim in these studies was to investigate how hypercapnia/acidosis modifies wound healing, these observations indicated that wound healing was mediated via NF-κB pathway activation [134]. In contrast, NF-κB inhibition with pharmacological inhibitors or with over-expression of IκBα-SR delayed epithelial wound closure. Although our current knowledge is limited on the role of the NF-κB pathway in lung repair, these initial data support the concept that NF-κB pathway is a "meeting point" of inflammatory and reparative processes in ARDS.

10.2.2 EVIDENCE FOR ROLE OF NF-κB IN MEDIATING THE EFFECTS OF HYPERCAPNIA

The aim of low stretch ventilation is to minimize injury to the lungs associated with mechanical ventilation. In this situation the acceptance of higher blood CO₂ levels, in order to achieve as minimal stretch/strain as possible, is accepted and is an integral part of the management of ARDS. It has been demonstrated by many that CO₂ is a potent biological agent and its main effect on the immune system seems to be inhibitory [302]. This is supported by a growing body of preclinical evidence that HCA can modulate – for benefit or harm – key aspects of injury and repair processes and
may constitute a double-edged sword in ALI/ARDS [42, 88, 89, 125-128, 130, 131, 133, 134, 139, 187].

10.2.2.1 Lung injury

The effect of HCA may be mediated, at least partially, via inhibition of the NF-κB pathway [See Chapter 1, section 1.4.7.2]. Takeshita et al first reported in in vitro work that HCA attenuates endotoxin induced injury via an NF-κB dependent mechanism [190]. The authors demonstrated that HCA inhibited NF-κB by preventing IκB-α degradation. This resulted in less inflammation, cell injury and reduced neutrophil adherence to the cell monolayer after LPS stimulation. Most recently, Wu et al demonstrated in an ex vivo ischaemia-reperfusion lung injury model that ventilation with 10% CO₂ attenuated lung injury and this was associated with significantly reduced NF-κB DNA binding activity, less nuclear NF-κB translocation and decreased IκB-α degradation and IKK phosphorylation in lung tissue [140]. Others also have shown that hypercapnia may inhibit the NF-κB pathway via facilitating the nuclear transport of IKKα, reducing the degradation of IκB-α in the cytoplasm and inhibiting the translocation of p65 to the nucleus [135]. Ten percent CO₂ blunted the expression of NF-κB dependent pro-inflammatory genes [CCL2, ICAM1, TNF-α], while anti-inflammatory genes, such as IL-10, were enhanced in vitro.

10.2.2.2 Lung Repair

Most recently our group have demonstrated that the potential for HCA to reduce pulmonary epithelial wound repair is also mediated via an NF-κB dependent mechanism [134]. HCA delayed wound closure by 50% in a dose dependent manner in three different lung epithelial cell lines in vitro. HCA also inhibited NF-κB activation, as evidenced by reduced κB luciferase expression and increased degradation of IκB-α compared to normocapnia. Interestingly, wound healing was independent from the MAP kinase pathway [main signal transduction pathway to activate NF-κB during injury] suggesting that other intracellular pathways might be involved in NF-κB activation during reparative processes than during injury. Our group also demonstrated that HCA reduced wound healing by a process involving decreased cell migration.
HYPERCAPNIC ACIDOSIS REDUCES THE SEVERITY OF PROLONGED SYSTEMIC SEPSIS

Systemic sepsis is one of the most common causes of ALI/ARDS and confers significant morbidity and mortality in the critically ill. Our group and others previously have demonstrated that HCA attenuates the hemodynamic effects of early septic shock and protected against sepsis induced ALI [131, 132, 187]. Given the serious consequences of potential immunosuppression arising from exposure to hypercapnia we investigated the effect of hypercapnia in septic rats exposed to 96 hours of environmental hypercapnia. We hypothesized that prolonged hypercapnia would worsen non pulmonary sepsis induced lung injury in rats. Given the central role of the NF-κB pathway in regulating injury and host defence in ARDS and sepsis, we also wished to investigate how hypercapnia would influence pulmonary NF-κB activity in septic animals.

PROLONGED CLP MODEL OF SYSTEMIC SEPSIS

The main advantage of the CLP model of sepsis is that it most closely replicates the nature and clinical course of intra-abdominal sepsis in humans [248-250, 287]. As we have discussed earlier [See Chapter 3, Section 3.2.2.1], the severity and outcome of injury after CLP can be tailored by altering the percentage of caecum ligated, by varying caecal puncture size and number, and also by introducing supportive measures, such as fluid administration [248, 253, 287, 288]. The main disadvantage of the CLP model is its variability in terms of sepsis severity and sepsis related mortality. We have used a stepwise approach to modify the above mentioned factors to achieve an acceptable mortality at 96 hours [See Chapter 4]. As we have shown in our pilot works, the main challenge imposed by this model was to find the right balance between sepsis severity, organ injury and survival. Our final 96h CLP model represented a low-mid grade sepsis with detectable, albeit mild, sepsis-induced ALI.

EFFECTS OF HYPERCAPNIA IN PROLONGED SYSTEMIC SEPSIS AND SEPSIS INDUCED LUNG INJURY

Sustained hypercapnia did not affect survival and hemodynamic response to systemic sepsis compared to normocapnia. However it did decrease serum lactate levels. Lung physiological indices and inflammatory markers, such as neutrophil count, TNF-α and
IL-6 levels, were not different between animals exposed to HCA versus normocapnia. Quantitative stereological analysis revealed a significant, but very modest reduction in acinar tissue fraction and increased acinar airspace volume fraction under HCA and this was associated with significantly less NF-κB DNA binding activity in lung tissue samples. There were no differences in BAL, blood and peritoneal fluid bacterial counts after 96 hours suggesting that prolonged hypercapnia did not alter bacterial proliferation or killing in this model.

10.3.3 MECHANISMS OF ACTION OF HYPERCAPNIA IN PROLONGED SEPSIS

Hypercapnic acidosis has been shown to improve global oxygenation and hemodynamic indices in various in vivo ALI models, including early and established sepsis [42, 130-132, 187, 335]. Improved cardiorespiratory performance, however, has been associated with better survival and lower lactate levels in severe sepsis induced ALI [131, 132]. The fact that in our experiments there was no difference in survival and haemodynamical indices between hypercapnic and normocapnic animals indicates that early fluid management may have obstructed the survival advantage of HCA in mild sepsis. The effect of hypercapnia on lung injury was moderate, resulting in significant but modest improvement in histological injury. The fact that pulmonary NF-κB activation was reduced under hypercapnic conditions provides another piece of evidence that the biological effect of hypercapnia is mediated partially via this key pathway. However, it is not clear whether the inhibition of NF-κB pathway in this context is good or bad. As we pointed out earlier, global NF-κB activity from lung tissue samples do not provide clear information concerning what are the activation profiles of the different cell types and what are the biological consequence of these. It has been shown that hypercapnic acidosis has a profound inhibitory effect both on the innate and adaptive immune system [136, 159-162, 164-166, 302]. Most recent data indicates that this effect may be mediated via NF-κB pathway inhibition [135-137]. The fact that bacterial growth was not increased under hypercapnic condition in the presence of reduced lung NF-κB activity is reassuring, but does not negate previous concerns regarding the potential harmful effects of hypercapnia in prolonged untreated bacterial pneumonia.
The protective effect of HCA in acute lung injury originated mainly from observations in non-septic models of ALI. In these contexts HCA appeared to reduce tissue injury due to its anti-inflammatory effect. Overall, in these experiments HCA was consistently associated with significant reduction in lung injury as well as reduction of lung inflammatory markers, such as neutrophil infiltration, and cytokine production [88, 89, 127-129, 138, 179]. However, immune competence is essential to an effective host response to microbial infection and overcoming sepsis. Hypercapnia and/or acidosis may modulate the interaction between host and microorganisms [see above 12.3.1.3] and may change the outcome of early and late bacterial sepsis. Currently available in vivo studies suggest that HCA reduces evolving and more established bacterial and systemic sepsis induced acute lung injury [42, 130]. In contrast, the effects of HCA in prolonged, untreated bacterial pneumonia seem to be harmful due to the immunosuppressive effect of HCA [133]. Our findings add more insights how prolonged exposure to hypercapnia may influence systemic sepsis and sepsis induced ALI. We demonstrated in an in vivo model that the beneficial effect on lung injury, albeit modest, was associated with reduced lung p65 activation, indicating that hypercapnia inhibited the NF-κB pathway. Importantly, hypercapnia did not increase bacterial load in BAL fluid and blood, providing some reassurance on the safety of hypercapnia in the context of systemic sepsis. As we have discussed earlier, the effect of hypercapnia may be mediated via inhibition of the NF-κB pathway. Most recent research indicates that inhibition of NF-κB pathway impairs host immune function and may be deleterious in the context of bacterial pneumonia [214, 215, 353]. Interestingly, NF-κB inhibition in pure endotoxin [211] or CLP sepsis induced lung injury models [210] is protective by reducing tissue injury caused by inflammation. Overall, these observations suggest that global inhibition of NF-κB pathway by hypercapnia may result in protection and harm depending whether the inflammation or the host immune response is suppressed more [Figure 10-1]. Importantly, the effect of hypercapnia may be further influenced by the origin of sepsis [i.e. direct or indirect pulmonary sepsis] or by the exposure time [i.e. short term vs. prolonged hypercapnia]. These observations highlight the need of more research on the interaction between hypercapnia/acidosis and the NF-κB
pathway and to see how this modifies inflammation and host immune response in diverse sepsis aetiologies and stages.

Figure 10-1 The putative effect of hypercapnia/acidosis in sepsis induced lung injury. The overall effect of hypercapnia may depend on the site of the injury. In direct lung injury the primary site of the injury is the lung epithelium while in systemic sepsis it is the lung endothelium. Increased NF-κB activation in these cells triggers neutrophil recruitment and amplifies the injury signal in various cells. This will activate inflammation and the host immune system resulting in tissue injury and bacterial clearance. Hypercapnic acidosis may block the NF-κB pathway and reduce tissue injury and host defence at the same time.

10.3.5 LIMITATIONS

There are number of limitations to our study. Our sepsis model was relatively mild, therefore to detect changes in physiological and inflammatory markers in response to moderate hypercapnia was difficult. The importance of injury severity in animal models has been shown in relation to other injury models, such as VILI [125],
ischaemia-reperfusion[127] and bacterial pneumonia induced ALI [42, 130, 141]. It is important to see that the absence of significant differences in injury markers between hypercapnia and normocapnia does not preclude that prolonged hypercapnia might have had ongoing effect on inflammation/immunity. Furthermore, we did not investigate the effect of hypercapnia after 96 hours, in the actual chronic phase of sepsis. Recent research in sepsis emphasises that some patients surviving the acute phase of sepsis may not recover but enter to a disease process which is featured by severe immunosuppression. The fact, that large body of evidence attests that both hypercapnia and acidosis has a profound inhibitory effect on the innate and adaptive immune system indicates that this area should be further explored.

10.3.6 CLINICAL IMPLICATIONS

Most of the recent data and our experimental findings in relevant preclinical models attest the safety and beneficial effect of hypercapnia in sepsis and sepsis induced ALI [42, 126, 130-132, 187]. However, in the setting of prolonged pneumonia, the immunosuppressive effect of hypercapnia remains a concern. The fact that the deleterious effect of hypercapnia can be reversed with antibiotic treatment in prolonged pulmonary sepsis highlights the importance of early appropriate empiric antibiotic treatment in sepsis [133]. Most of the success of intensive care medicine in sepsis is related to the management of early septic shock [285]. Although, many patients survive this phase and recover, the majority of deaths contributing to the mortality in sepsis occur in patients who develop immunoparalysis during their stay in the intensive care unit [301, 310]. This phase is mainly described with secondary or hospital acquired infections including virulent organism, opportunistic infections or reactivation of latent organisms, such as cytomegalovirus or herpes simplex virus [286]. Prolonged ventilation, even with low tidal volume, and sustained hypercapnia may impose additional risks in this patient population. To prevent potential adverse effects of hypercapnia in sepsis, alternative ventilation strategies, such as HFOV, ECMO or lung assist device [NOVALUNG] may help to correct prolonged respiratory acidosis and reduce ventilation induced lung injury in patients with severe ARDS, minimizing the harmful effects of conventional mechanical ventilation.
10.4 HYPERCAPNIC ACIDOSIS ATTENUATES VENTILATION INDUCED LUNG INJURY

10.4.1 HIGH STRETCH MODEL OF VILI

Our primary aim was to develop a high mechanical stretch model which incorporated the main physiological and inflammatory alterations seen in human ventilation induced lung injury. We had to take into consideration that we may have to use excessive injury in order to detect the effect of hypercapnia in physiological parameters. In our pilot work, we identified few factors that influenced injury severity as well as model variability. By standardizing the age of the animals and using animals strictly from the same batch in each experimental series we could control reasonably for model variability [See Chapter 6]. To balance between injury severity and survival, a very similar situation we encountered during the CLP model development, we needed to determine the threshold pressure and subsequently modify the respiratory rate to obtain the desired two VILI models with distinct severities. According to our observations, the exponential nature of injury in response to increasing PIP makes it difficult to relate lung injury severity to the degree of mechanical stretch. The fact that the effect of respiratory rates on physiological lung injury manifests above the threshold pressure allowed us to develop a moderate and severe VILI model. Although the difference between respiratory rates [15 vs. 18] in the two models seems modest, we could reliably demonstrate that this was indeed associated with a very distinct injury response to high stretch. Furthermore, our observations were supported by previous studies in the literature showing that RR may independently influence injury severity and survival in animals, particularly when the inspiratory pressure is high [325-330].

In summary, we have developed an in vivo rodent model that was suitable to measure the effect of HCA on VILI at two distinct levels of injury severity. We took into consideration controllable factors, such as age and batch, and also determined the optimal injury time and severity to reliably demonstrate the main characteristics of acute lung injury.
10.4.2 EFFECTS OF HYPERCAPNIA IN MODERATE AND SEvere VILI

Along with previous reports [88, 89, 125, 138], we confirm that HCA attenuates VILI. However, the protective effect of hypercapnic acidosis was more obvious when lungs were exposed to severe than moderate mechanical stretch. This may relate to the fact that the physiological effect of HCA greatly depends on the severity of the lung injury model. The protective effect of HCA in moderate VILI has few important clinical implications: (1) The anti-inflammatory effect of hypercapnia may be present in situations when ALI is not associated with gross physiological changes. As we mentioned earlier [See Chapter 7], this may have an importance in light of Kregenow et al’s findings, namely that permissive hypercapnia was not beneficial in patients ventilated with protective ventilation strategy [69]. (2) Despite best efforts to minimise VILI by applying lung protective ventilation, some lung areas can be subjected to significant overdistension in ALI/ARDS due to regional heterogeneity [53, 54]. Protection of over-distended regional units even when the lung does not seem to be grossly injured may hold the key for prevention of further injury associated with mechanical ventilation.

10.4.3 MECHANISM OF ACTION OF HYPERCAPNIA

The potential of HCA to modify key aspects of lung injury [190, 140] and repair [134] processes via inhibition of the NF-κB pathway has been increasingly recognised. This is important, since a number of strands of evidence suggest that the NF-κB transcriptional pathway might be one of the common pathways coordinating injury, defence and reparative processes in ALI/ARDS [216].

Our results extend the knowledge regarding the effect of HCA on the NF-κB pathway in the setting of ventilation induced lung injury. High mechanical stretch increased NF-κB activation in lung tissue as evidenced by decreased cytoplasmic levels of the inhibitory molecule IκB-α. In the moderate VILI series HCA significantly attenuated stretch induced IκB-α degradation compared to normocapnia, and resulted in similar levels of cytoplasmic IκB-α content as in sham, non-ventilated animals. Nuclear p65 concentration was also reduced by hypercapnia, representing decreased NF-κB translocation. These changes occurred in parallel with reduced NF-κB dependent
cytokine release in BAL samples. Although, there are several limitations to our
observations, this is the first time that it has been demonstrated in an in vivo VILI
model that the anti-inflammatory effect of HCA is mediated via inhibition of the NF-κB
pathway. This piece of information may contribute to elucidate the role of permissive
hypercapnia in the management of ALI/ARDS, help to minimize the harmful and
promote the beneficial aspects of HCA.

10.4.4 LIMITATIONS

There are a number of limitations to be considered regarding our experiments [See in
more detail in Chapter 7, Section 7.4.2.2]. However, these limitations highlight a few
interesting points and may help to address future research directions. Firstly: we used
high airway pressure and zero PEEP to cause VILI to healthy lung. As we mentioned
earlier, when we relate this to clinical observations we can see that the applied
pressure [30 cmH_2O] and stretching forces may be relevant in patients with ARDS. We
know from CT imaging studies [52] that regional over-distension and atelectrauma is
ongoing even during lung “protective” ventilation. Therefore, it is possible that the
beneficial or harmful effects of HCA are present at regional levels even when the
inflating pressures/volumes are relatively low. Secondly: we added 5% CO_2 to the
breathing circuit to test the effect of HCA on VILI. The rationale for this is that it is not
possible to model injurious ventilation [high intensity ventilation] and low alveolar
ventilation associated hypercapnia at the same time. Adding CO_2 to the breathing
circuit may imply an entirely different alveolar CO_2 distribution [probably more
homogenous] than permissive hypercapnia which is the result of alveolar hypoventilation. We do not know in fact what the regional alveolar pCO_2 and pH values
are in the heterogenously injured lung in real life. It is possible, that some alveoli are
over-ventilated and hypocapnic, some of them are normocapnic, or that some have
very high CO_2 tensions. The overall impact of these differences is unknown and still
needs to be elucidated. Thirdly: we used an in vivo VILI model to demonstrate the
effect of HCA on the NF-κB pathway. Although, it is clear that intracellular pathways
are best studied in in vitro models, our results from the in vivo experiments provided a
good estimate how HCA may influence the NF-κB pathway globally. These insights,
however, helped to design a series of experiments using an in vitro VILI model. Ansari
B demonstrated that both moderate [5%], and more severe [10%] HCA reduced stretch-induced NF-κB activation in lung epithelial cells and reduced IL-8 production [335]. Although this obviously does not fully explain the in vivo situation, it confirms our findings, and provides another piece of evidence on the biological mechanism of HCA in VILI.

10.4.5 CLINICAL IMPLICATIONS

Permissive hypercapnia, as part of protective ventilation, is regularly used in intensive care management in patients with ARDS. The fact that high mechanical stretch and hypercapnia and/or acidosis may act and share their effect on the same intracellular pathway provides an opportunity to minimize the harmful effects of mechanical ventilation in ARDS patients. The potential for permissive hypercapnia to inhibit the NF-κB pathway and reduce inflammation may counterbalance stretch induced injury. This may be beneficial at the initiation of mechanical ventilation. At later stages, however, direct and cell specific modification of NF-κB activity may gain more importance in order to harness the beneficial or cancel the deleterious effects of permissive hypercapnia. More understanding is needed as to how permissive hypercapnia affects NF-κB activity and what are the implications of these effects in diverse clinical contexts.

10.5 INHIBITION OF NF-κB ACTIVITY VIA GENE THERAPY ATTENUATES VILI

10.5.1 RATIONALE FOR GENE BASED APPROACHES TO MODULATE NF-KB

High stretch mechanical ventilation can directly cause ARDS in the healthy lung and can worsen pre-existing ARDS. In contrast, low stretch mechanical ventilation has been shown to reduce mortality in ARDS [5]. To date there are no existing specific therapies that would have significant impact on mortality in this patient population [See Chapter 1, Section 1.2.2.1]. Alternative approaches, such as gene delivery, may present a unique opportunity to explore or modify key pathways by over-expressing or inhibiting genes central to ARDS [218].
10.5.1.1 Direct therapeutic potential of NF-κB inhibition

Providing that NF-κB activation is central to the initiation and coordination of inflammation in various lung injury aetiologies, inhibition of this pathway could represent an attractive therapeutic approach. There is considerable *in vivo* preclinical evidence that blocking NF-κB reduces lung injury related to ischaemia-reperfusion [213], endotoxin [211] and CLP sepsis [210]. We have also demonstrated that NF-κB inhibition by hypercapnia blunted mechanical stretch induced ALI [335]. It is also clear that to achieve beneficial outcome, inhibition of this pathway may have to be cell specific. The primary injury in ALI is directed either to the lung epithelium or lung endothelium. Protecting these cells and reducing their NF-κB responsiveness may help to limit injury signals and to prevent the propagation of tissue damage. From this point of view, the lung is an ideal target organ. *Firstly*: genetic materials can be easily administered *via* the tracheal or intravenous route. *Secondly*: cell specific and targeted, i.e. epithelial, endothelial or both, administration of transgenes is also possible and has been demonstrated in various *in vivo* ALI models [241-244, 246]. Due to rapidly developing gene technologies and the fact that gene therapy is a real therapeutic option in many other medical conditions [See Chapter 8, Section 8.2.2], targeted and specific gene delivery in ARDS has realistic and promising foundations.

10.5.1.2 Elucidation of mechanistic role of NF-κB in VILI

The difficulty in studying intracellular pathways *in vivo* relates to the complexity of whole animal model. The influence of ongoing interactions between cells and organs often interferes with the detection of the biological signal or signals one intends to investigate. Therefore it may be difficult to establish casual relationships or drawing valid inferences as to how certain interventions affect intracellular pathways. However, *in vivo* models are indispensible in translational research and ultimately they are essential to bring scientific observations and therapeutic options to the clinical sphere. Gene delivery may be a powerful tool to study intercellular pathways, such as the NF-κB pathway, and their contribution in the pathomechanism of VILI. Targeted activation or inhibition of various steps of the NF-κB pathway, i.e canonical and non-
canonical pathways, may help to elucidate the diverse regulatory mechanisms and their contribution in the pathomechanism of mechanical stretch induced lung injury.

10.5.2 IκB-α-SUPER-REPRESSOR AS A KEY PATHWAY INHIBITOR

Regulatory elements act at diverse levels along the NF-κB signal transduction pathway, including membrane receptors, adaptor molecules, IKK complexes, IκB proteins, NF-κB dimers and the posttranslational apparatus [200, 201]. However, the key step for controlling NF-κB activity and signal responsiveness is the interaction between IκB-α and NF-κB [202]. The inactive NF-κB homo/heterodimer resides in the cytoplasm in association with the IκB-α molecule. IκB-α phosphorylation at two serine residues [Ser32 and Ser36] by the IKK complex initiates the degradation of this inhibitory molecule and the activation of NF-κB. There are several ways to inhibit this rate limiting step, including pharmacological inhibitors [PDTC; BAY 11-7085] or over-expressing proteins which prevents IκB-α degradation. IκBα-super-repressor [IκBα-SR] protein is a mutant form of the original IκB-α, in which the two target serine residues [Ser32 and Ser36] are mutated to alanines. Over-expressed IκBα-SR in a cell cannot be phosphorylated by the IKK complex and therefore it does not degrade. The end result is the prevention of nuclear translocation of NF-κB and inhibition of NF-κB dependent gene expression. IκBα-SR transgene delivery to various cell lines and to living animals has been used to investigate the role of the NF-κB pathway in various diseases processes. The rationale to use IκBα-SR transgene to investigate how NF-κB activity influences the inflammatory response in mechanical stretch induced lung injury is clear, given the central role of the NF-κB pathway in acute lung injury. Although IκB-α-SR has been used in *in vitro* stretch induced VILI models [335], it has not been tested in *in vivo* ventilation induced lung injury. Our aim was to see how NF-κB inhibition *via* pulmonary IκBα-SR overexpression would affect the course of VILI providing a great opportunity to gain insights into a potential gene therapy and to the pathomechanism of ventilation induced ALI.

10.5.3 EFFECTS OF IκBα-SR OVEREXPRESSION IN INJURY SEVERITY IN VILI

We hypothesized that inhibition of the NF-κB pathway by over-expressing IκBα-SR would reduce VILI. AAV6-IκBα-SR pre-treatment did not affect survival, hemodynamic
response or serum lactate levels in response to injurious ventilation compared to vehicle and AAV6-Null pre-treatment. Arterial oxygenation and lung mechanics were comparable among the groups. Importantly, arterial O\textsubscript{2} tension on FiO\textsubscript{2}:1.0 was lower, and AaDO\textsubscript{2} was higher in animals treated with AAV6-IκBα-SR and this was associated with significantly less BAL protein content. Inflammatory markers and histological injury did not differ among the groups. We also tested if transgene delivery was effective. Flow cytometry analysis showed a \textasciitilde21.4\% lung epithelial transduction rate and this was associated with 21 fold IκBα-SR gene expression in transduced animals. Although these results could not fully support our hypothesis, it provided valuable insights and preliminary data for further experiments. Subsequent changes in AAV6-IκBα-SR constructs led to better transduction efficiency and obvious protective effects in VILI. Most recently, our group demonstrated that AAV6-IκBα-SR pre-treatment significantly improved survival and arterial oxygenation, reduced BAL neutrophil infiltration and histological injury in \textit{in vivo} VILI [Hayes M et al, unpublished data]. Furthermore, transfection of lung epithelial cells [A549] with IκBα-SR transgene significantly reduced NF-κB activation and inflammation in an \textit{in vitro} mechanical stretch induced lung injury model [335]. Overall, this data supports the concept that NF-κB pathway activation is central to mediating the inflammatory effects of high mechanical stretch induced lung injury.

10.5.4 LIMITATIONS

There are several limitations to our experiments. As we discussed in Chapter 9, we did not carry out a dose response study to define the optimal dose for the vector-transgene construct due to the excessive difficulty in producing large amounts of viral vector-transgene stock [Chapter 8]. However, \(10^{10}\) AAV6-IκBα-SR administration in subsequent experiments resulted in NF-κB inhibition and attenuation of VILI in rats [Hayes M et al, unpublished data]. Furthermore, a \textasciitilde21.4\% transduction rate in lung epithelial cells and 21 fold IκBα-SR overexpression may have not resulted in sufficient transgene expression to exert protective effect in VILI. Alternative pulmonary gene delivery methods, such as nebulizers, may have been more advantageous and would have provided more homogenous vector distribution than intratracheal instillation. The drawbacks of our experiments and difficulties encountered during viral vector
construction however point toward few, ongoing technical issues in gene therapy: (1) importance of designing optimal vector constructs and to optimize scale up viral stocks for in vivo delivery; (2) overcoming difficulties related to the extracellular barrier function of the lung tissue; (3) to prevent the potential host immune activation in response to viral vector delivery; (3) achieving reliable tissue or cell specific vector delivery; (4) improved transgene expression efficiency and controllability over time.

10.5.5 CLINICAL IMPLICATIONS

Gene therapy for ARDS is a viable option, given the ease of access to the lung. However, there are several key questions that need to be addressed and resolved before gene therapy for ALI/ARDS can become a realistic therapeutic intervention. We need to know which genes or set of genes are the ideal target in the lung to ameliorate ARDS. This may vary depending on the aetiology of the lung injury and the stage of the disease process. We know from accumulating preclinical data that inhibition of inflammatory pathways coordinating gene expression, such as the NF-κB pathway, may be beneficial in the acute inflammatory phase of ALI while it can be potentially harmful in lung repair. This may require vector constructs which can be switched on/off depending on the phase of the disease process. The fact that the lung epithelium is very easily available in patients on mechanical ventilator may help to administer protective genes right at the start of mechanical ventilation and minimize the harmful effects of stretch related lung injury.

10.6 HYPERCAPNIA, ARDS AND THE NF-κB PATHWAY – AN INTEGRATED PARADIGM

10.6.1 HYPERCAPNIA AND SEPSIS – BENEFIT OR HARM?

The current state of knowledge on the effect of hypercapnia/acidosis in sepsis induced lung injury supports two important insights: (1) hypercapnic acidosis reduces tissue injury caused by the inflammatory response; (2) prolonged exposure to hypercapnia reduces bacterial clearance and worsens bacterial pneumonia. While a large body of in vivo evidence supports the first observation in early [42, 131], established [130, 132], and prolonged sepsis [our findings][131], the second observation has been suggested by one in vivo experiment[133]. The link between the biological effect of hypercapnia and NF-κB pathway inhibition in sepsis has been demonstrated in several in vitro
experiments [135, 136, 190] and in *Drosophila* [137]. These experiments suggest in one hand that NF-κB inhibition mediated by hypercapnia reduces inflammatory cytokine response in sepsis, but on the other hand it reduces host defences by reducing macrophage phagocytic activity and production of antimicrobial peptides necessary for *Drosophila* to survive during bacterial infection [See section 10.2.2.1]. Our findings in a prolonged sepsis induced ALI model are the only *in vivo* data to date supporting the NF-κB mediated anti-inflammatory effect of hypercapnia.

The fact that hypercapnia and/or acidosis inhibit the host immune system via the NF-κB pathway may have serious consequences in septic patients requiring mechanical ventilation. It is clear that HCA is beneficial in septic and non-septic ALI when the host immune response is the major contributor to tissue injury. However, in bacterial sepsis-induced lung injury, when the final outcome principally depends on the successful elimination of pathogen microorganisms, hypercapnia may be deleterious by inhibiting bacterial clearance. Of concern, severe sepsis-induced organ failure is the leading cause of death in critically ill patients and sepsis-induced ARDS is associated with the highest mortality rate. Infective complications in non-septic intensive care patients are very common, with a prevalence of 44%. In these patients, dysregulated inflammation can easily lead to excessive host damage, protracted infection and dissemination of the disease resulting in MOF and death. Therefore, the possibility of hypercapnia to exert its beneficial and harmful effects on NF-κB inhibition highlights the importance of these research areas and the need for more *in vitro, ex vivo* and *in vivo* sepsis studies.

### 10.6.2 HYPERCAPNIA REDUCES THE SEVERITY OF VILI

It has been repeatedly demonstrated that HCA attenuates VILI by inhibiting inflammation. In spite of the applied low tidal volumes, lung protective ventilation may cause further damage to the lungs in mechanically ventilated patients. Our findings add another piece of evidence to previous preclinical observations that HCA may be protective in less severe mechanical ventilation induced ALI. The importance of this is highlighted in the fact that *Kregenow et al* could not show mortality benefit in the low tidal volume group in the secondary analysis of the ARMA trial [69]. It is a well
established concept that mechanical ventilation causes biotrauma [86, 101]. High stretch injury causes gene expression and inflammation by activating transcription factors, such as NF-κB. Our experiments confirmed and demonstrated that the biological effect of HCA was mediated by NF-κB inhibition, indicating that permissive hypercapnia may attenuate injuries associated with mechanical ventilation. The long term effect or prolonged exposure to permissive hypercapnia is unknown and there is some data to indicate that hypercapnia may delay the reparative processes of ARDS [134]. The application of gene delivery, as it has been demonstrated in our last experimental series, may offer a unique possibility to dissect the contribution of the NF-κB pathway to the effects of HCA. This approach may help to modify NF-κB pathway activity in the presence of lung protective ventilation and optimize the balance between inflammatory and reparative processes and minimize and/or maximize the biological effect of permissive hypercapnia in patients with ARDS.

10.6.3 ROLE OF INHIBITION OF NF-κB IN MEDIATING THE EFFECTS OF HCA

10.6.3.1 HCA reduces lung injury severity via NF-κB inhibition

The potential of permissive hypercapnia to minimize the harmful effects of mechanical ventilation and other non-septic ALI by modulating pathways key to lung injury, such as the NF-κB pathway, may facilitate recovery from ALI/ARDS. Interesting insights to the interaction between hypercapnic acidosis and the NF-κB pathway have emerged over the last decades. One of the putative mechanism by which hypercapnia attenuates inflammation is realized by inhibition of the rate limiting step of the NF-κB pathway. Hypercapnic acidosis has been shown to inhibit IκB-α degradation in various \textit{in vitro} and in one \textit{ex vivo} ALI models [134, 140, 190, 335]. Our work has demonstrated first in an \textit{in vivo} model that HCA attenuated VILI and this was mediated by inhibition of the NF-κB pathway. \textit{Ansari B}, from our research group extended and confirmed this observations in an \textit{in vitro} stretch model [335]. Recently, \textit{Wu et al} demonstrated that pulmonary ischaemia-reperfusion ALI was significantly attenuated by hypercapnic acidosis and this was mediated via pulmonary NF-κB inhibition [140]. These initial observations suggest that the beneficial, anti-inflammatory effects of HCA may be
realized via NF-κB inhibition. This concept however needs to be tested in other non-septic and septic ALI models before it can be exploited in clinical practice.

**10.6.3.2 HCA slows lung repair via NF-κB inhibition**

Healthy repair processes are essential to successful recovery after ALI/ARDS. Interventions or clinical states that disturb these processes may lead to increased morbidity and mortality following ARDS. Long term follow-up studies have shown that, while pulmonary function improves 3-6 months after ARDS, most survivors exhibit a certain degree of restrictive impairment and reduced diffusion capacity equivalent with moderate lung fibrosis [10, 12, 13]. Prolonged exposure to protective lung ventilation and permissive hypercapnia may hinder tissue repair and may contribute to the dysregulation of reparative processes in ALI/ARDS. This concept has been suggested first by Doerr et al [189] demonstrating that HCA has a significant contribution to impaired epithelial membrane healing after VILI. Recently, our group suggested that hypercapnia reduces lung epithelial wound repair via inhibition of the NF-κB pathway [134]. Other potentially harmful aspects of impaired epithelial repair, although not supported by scientific evidence, is the possibility of aggravation of biotrauma by allowing/maintaining the “leak” of inflammatory mediators to the systemic circulation causing distal organ injury. Further studies are needed in relevant in vivo models to explore the relationship between HCA, the NF-κB pathway and lung repair.

**10.6.3.3 Role of inhibition of NF-κB in sepsis induced lung injury**

The potential of hypercapnia to inhibit the immune system and to attenuate host immune system is a real concern. Helenius et al showed in Drosophila that moderate hypercapnia [7-13%] inhibited a large array of genes responsible for antimicrobial peptide production [137]. These proteins are essential to mediate host immune responses to bacterial infection in Drosophila. Exposure to hypercapnia increased the susceptibility and mortality of Drosophila to various Gram negative and positive bacteria, such as E. coli, E. faecalis, S. aureus. The authors also suggested that hypercapnia inhibited the NF-κB pathway. However, this may have occurred via other pathways than IkB-α degradation [i.e. downstream from NF-κB activation or by direct
inhibition of the transcriptional apparatus]. Wang et al and Cummins et al confirmed and extended these observations in *in vitro* LPS stimulated macrophages and 6 other laboratory cell lines [135, 136]. The common findings in these studies were that moderate hypercapnia inhibited macrophage phagocytosis and inflammatory gene expression via NF-κB inhibition. This involved on one hand reduced IκB-α degradation, but also an IκB-α degradation independent pathway. Interestingly, these effects were independent from pH and MAP kinase pathway. Observations by our group in an *in vitro* wound repair model were partially similar to these [134]. Hypercapnia inhibited the NF-κB pathway and wound repair, however this seemed to be the effect of CO₂ rather than pH and was MAP kinase pathway independent. These data highlights that the effect of respiratory acidosis on the NF-κB activity and host response is complex and may be directed to multiple levels at the NF-κB pathway or may involve other intracellular pathways. In our work, we demonstrated that prolonged hypercapnia reduced pulmonary p65 DNA binding in septic animals providing another piece of evidence on the inhibitory effect of hypercapnia/acidosis on the NF-κB pathway. Although, this inhibition was associated with better histological scores and, reassuringly, with no changes in bacterial load compared to normocapnia, it does not preclude the potentially deleterious effects of HCA in sepsis. The fact that hypercapnia and/or acidosis is a potent immunosuppressive agent and may promote bacterial growth in mechanically ventilated patients is worrisome [186]. These data may imply that both the benefits and harms of HCA are NF-κB dependent and therefore NF-κB inhibition may constitute a double-edged sword in ALI/ARDS. Given the broad influence of NF-κB activation on cell function, more understanding how NF-κB pathway regulates inflammation and repair are needed.

10.6.4 AREAS OF UNCERTAINTY AND FUTURE RESEARCH DIRECTIONS

There are several questions in the role of hypercapnia and/or acidosis in lung inflammation and its relation to the NF-κB pathway. This can be explored best if we try to integrate the currently available observations on this area. As it is outlined in Figure 10-2, ALI/ARDS of various aetiologies activate the NF-κB pathway and initiate gene expression in multiple cell types in the lung. Gene expression profile and dynamics however may depend on the origin of the specific insult, i.e. septic vs. non-septic or
direct vs. indirect ALI. The biological response to NF-κB activation in each individual cell is also determined by its role and function in the lung. Furthermore, the evoked biological responses to NF-κB activation - such as pro/anti-inflammatory responses, innate/adaptive immune response and reparative processes – may run in parallel over time. Therefore there is a real potential to cause both harm or benefit by blocking the NF-κB pathway by hypercapnia/acidosis. Uncertainties and future directions are related to the following areas:

(1) **Sepsis vs. non-septic ALI/ARDS.** While we have large body of evidence on the protective effect of hypercapnic acidosis in non septic ALI models the potentially harmful effect of hypercapnia in the setting of sepsis induced ALI has been recently emerged. We need to further explore the immunomodulatory effect of hypercapnia and/or acidosis in late sepsis by using more relevant prolonged sepsis models. Although preclinical studies indicate that hypercapnia and/or acidosis inhibits the NF-κB pathway, these are very scarce and limited to mainly *in vitro* and *ex vivo* studies. The hypothesis that the effects of hypercapnia is related to NF-κB inhibition needs to be further tested in various *in vivo, ex vivo* non-septic and septic ALI models and humans.

(2) **Direct vs. indirect ALI/ARDS.** Our findings that hypercapnia did not worsen sepsis induced lung injury, while it is clearly harmful in the setting of bacterial pneumonia, directs our attention to the fact that direct or indirect ALI may be different entities involving distinct intracellular processes and may require different therapeutic approaches. This concept has been advocated by Gattinoni and also supported by others [See Chapter 1, section 1.1.3.1]. Currently, the effects of hypercapnia and the NF-κB pathway in these contexts are unclear and need further exploration.

(3) **CO₂ vs. pH and the NF-κB pathway.** The question whether the biological effect of respiratory acidosis is the result of CO₂ or pH is controversial. The difficulty of this question lies in the fact that the methodological approaches by which experimental studies tried to separate the effect of CO₂ from pH varies largely. Buffering respiratory acidosis can be performed in different ways, resulting in substantially different and controversial observations [See Chapter 1, section 1.4.7.1]. Most recent studies on the
effects of hypercapnia and/or acidosis on the NF-κB pathway highlighted several interesting points. Hypercapnic acidosis inhibited IκB-α degradation in *in vitro* endotoxin [190], *in vitro* and *in vivo* VILI [335] and *ex vivo* pulmonary ischaemia-reperfusion injury [140]. In contrast, IκB-α degradation and delayed wound repair were related to CO₂ rather than to pH [134]. Others have suggested that the immunosuppressive effect of CO₂ may also relate to an intracellular CO₂ sensor which inhibits NF-κB directly, independently from IκB-α degradation [135]. This data indicates that hypercapnia/acidosis may act on different pathway/s or regulatory steps of the NF-κB pathway which may convey distinct biological effects.

(4) **Repair in ALI.** Reparative processes in ALI in the context of hypercapnia and the NF-κB pathway have not been investigated *in vivo* due to lack of relevant animal models. Recently, our group has developed an *in vivo* VILI repair model in rats [361]. This model reliably replicates normal lung repair after high stretch lung injury and may provide an excellent tool to further explore the biological effects of hypercapnia. Integration of *in vitro* data with *in vivo* observations may help to elucidate the role of hypercapnia in the reparative phase of ALI/ARDS.

(5) **Gene therapy and ARDS.** We need to know which gene or set of genes are the ideal targets in the lung to ameliorate ARDS. We know from accumulating preclinical data that modulation of inflammatory pathways coordinating gene expression, such as the NF-κB pathway, might be one of the options. However, more work needs to be carried out to determine how these pathways are integrated and what is the role of these in the development and resolution of ARDS.
INJURY

Septic vs. Non-Septic ARDS
Direct vs. Non-Direct ARDS

NF-κB activation

Gene expression

Endothelial cells
Epithelial cells
Immune cells
Interstitial cells

Pro-inflammatory response ↑
Anti-inflammatory response ↑
Innate immune response ↑
Reparative processes ↑

CO₂ / H⁺

Figure 10-2 Integrated overview of injury processes in ARDS and the inhibitory effect of hypercapnia and/or acidosis. ALI/ARDS of various aetiologies activate the NF-κB pathway and initiate gene expression in multiple cell types in the lung. Gene expression profile and dynamics however depend on the specific insult and its magnitude. The biological response to NF-κB activation in each individual cell is also determined by its role and function in the lung. NF-κB activation results in increased: pro/anti-inflammatory responses, innate/adaptive immune response and reparative processes. The overall balance/imbalance of these processes will determine outcome. Blocking the NF-κB pathway by hypercapnia/acidosis would affect all biological responses and may be harmful or beneficial in certain stages of ARDS.
10.7 SUMMARY

The work presented in this thesis intended to investigate:

1. The safety of permissive hypercapnia in the context of sepsis and sepsis induced ALI and how NF-κB is mediating these effects.

2. The role of the NF-κB pathway in mediating the effects of hypercapnic acidosis in VILI.

3. The role of NF-κB inhibition via gene delivery to explore the pathomechanism of VILI and to exploit the potential of gene therapy to minimize the harmful effects of mechanical ventilation.

10.8 CONCLUSIONS

1. Sustained hypercapnic acidosis did not worsen sepsis and, in fact, attenuated sepsis induced lung injury in an in vivo rat model of CLP. Hypercapnic acidosis reduced p65 activity in lung tissue suggesting that the protective effect of HCA may have been related to the inhibition of the NF-κB pathway. Sustained hypercapnic acidosis did not increase bacterial load compared to normocapnia in CLP sepsis.

2. Hypercapnic acidosis is protective against moderate and severe VILI in an in vivo rat model. The protective effect of HCA appeared to be mediated via NF-κB pathway inhibition. Hypercapnic acidosis reduced IκB-α degradation in the cytoplasm and reduced nuclear p65 translocation in lung samples in VILI.

3. Intrapulmonary delivery of AAV6-IκBα-SR conferred limited protection against VILI in an in vivo rat model. This was possibly related to suboptimal gene expression in lung tissue. AAV6 vector delivery with or without transgene did not cause lung injury reassuring the safety of this vector system.
CHAPTER 11
CHAPTER 11 PUBLICATIONS AND PRESENTATIONS ARISING FROM WORK

11.1 PEER REVIEWED PUBLICATIONS

1. “Hypercapnic acidosis attenuates ventilation induced lung injury by an NF-κB dependent mechanism.”
   Contreras M*, Ansari B*, O’Toole D, Higgins BD, Curley GF, Hassett P, Laffey JG.
   Crit Care Med [*Joint first authors].

   Costello J, Higgins BD, Contreras M, Chonghaile MN, Hassett P, O’Toole D, Laffey JG.
   Crit Care Med. 2009 Aug;37(8):2412-20

3. “Differential effects of buffered hypercapnia versus hypercapnic acidosis on shock and lung injury induced by systemic sepsis.”
   Higgins B, Costello J, Contreras M, Hassett P, O’Toole D, Laffey JG.
   Anesthesiology. 2009 Dec;111(6):1317-26

11.2 REVIEW PAPERS AND BOOK CHAPTERS

1. “Hypercapnia – Permissive, Therapeutic or not at all?”
   Hassett P, Contreras M, Laffey JG

2. “Is permissive hypercapnia helpful or harmful?”
   Contreras M, Hassett P. Laffey JG
   Evidence Based Practice of Critical Care. 2010, Elsevier. Deutschman CS and Neligan PJ.

3. “Hypercapnia and acidosis in sepsis: a double-edged sword?”
   Curley G, Contreras M, Nichol AD, Higgins BD, Laffey JG.
4. “Clinical review: gene-based therapies for ALI/ARDS: where are we now?”

Devaney J, Contreras M, Laffey JG.


11.3 PUBLISHED ABSTRACTS

1. “Hypercapnic acidosis attenuates the development of septic shock following cecal ligation and puncture”

Costello JF, Contreras M, Higgins BD, O’Toole D, Laffey JG.


2. “Buffered hypercapnia attenuates the development of septic shock in systemic polymicrobial sepsis”

Contreras M, Higgins BD, Costello J, Ni Chonghaile M, Laffey JG.

European Society of Intensive Care Medicine, 21st Annual Congress, Lisbon 21-24 September 2008

3. “Prolonged hypercapnia reduces the severity of acute lung injury in chronic polymicrobial sepsis”

Contreras M, Costello J, Higgins B, O’Tool D, Hassett P, Laffey JG.


4. “Hypercapnic acidosis attenuates moderate and severe ventilator induced lung injury by an NF-κB dependent mechanism”

Contreras M, O’Toole D, Higgins B, Ansari B, Curley G, Masterson C, Laffey JG

The American Thoracic Society Meeting, New Orleans, 14-19th May 2010

5. “Hypercapnic acidosis impairs inflammatory signalling via inhibition of multiple points of the NF-κB pathway.”
D. O’Toole, C. Masterson, A.M. Leo, M. Contreras, P. Hassett, J.G. Laffey


11.4 ORAL PRESENTATIONS

1. “Buffered hypercapnia attenuates the development of septic shock in a rodent model of systemic polymicrobial sepsis”.

Contreras M, Higgins BD, Costello JF, Ni Chonghaile M, Laffey JG

Western Anaesthesia Symposium. Westport 4-6 April 2008

The Western Anaesthetic Symposium is the annual scientific meeting organised by the Department of Anaesthesia and Intensive Care, University College Hospital Galway and attracts audience and speakers from Ireland and abroad.

2. “Buffered hypercapnia attenuates the development of septic shock in systemic polymicrobial sepsis”

Contreras M, Higgins BD, Costello J, Ni Chonghaile M, Laffey JG.

European Society of Intensive Care Medicine. 21st Annual Congress, Lisbon 21-24 September 2008

3. “Hypercapnic acidosis attenuates inflammatory response in ventilator induced lung injury by inhibition of the NF-κB pathway”.

Contreras M, Curley G, Higgins B, O’Toole D, Ansari B, Laffey JG

11th Current Controversies in Anaesthesia and Peri-operative Medicine Conference. Dingle, 14-17th October 2009

4. “Hypercapnic acidosis attenuates excessive mechanical stress induced ALI by an NF-κB dependent mechanism”.

Contreras M, Ansari B, Curley G, O’Toole, Higgins B, Laffey JG

Western Anaesthesia Symposium, Westport 16-17th April 2010

5. “Hypercapnic acidosis inhibits NF-κB mediated inflammation in a rodent model of ventilator induced lung injury”
6. “Hypercapnic acidosis may attenuate moderate and severe ventilator induced acute lung injury by an NF-κB dependent mechanism

Contreras M, Ansari B, Curley G, O’Toole D, Laffey JG

Delaney Medal Competition, College of Anaesthetists, Dublin, 3rd March 2011

The Delaney medal is a prestigious memorial medal awarded by the Council of the College of Anaesthetists annually for research either clinical or laboratory based in the field of Anaesthesia and Intensive Care. Six presenters from Ireland are shortlisted each year for their work.

11.5 POSTER PRESENTATIONS

1. “Hypercapnic acidosis attenuates the development of septic shock in a rodent model of systemic polymicrobial sepsis”

Contreras M, Costello JF, Higgins BD, O’Toole D, Hassett P, Laffey JG.

British Association for Lung Research Summer Meeting, NUI Maynooth 5-7 September 2007

2. “Prolonged hypercapnia reduces the severity of acute lung injury in chronic polymicrobial sepsis”

Contreras M, Costello J, Higgins B, O’Tool D, Hassett P, Laffey JG.


3. “Hypercapnic acidosis attenuates moderate and severe ventilator induced lung injury by an NF-κB dependent mechanism”

Contreras M, O’Toole D, Higgins B, Ansari B, Curley G, Masterson C, Laffey JG

The American Thoracic Society Meeting, New Orleans, 14-19th May 2010
11.6 PRIZES

1. “Buffered hypercapnia attenuates the development of septic shock in a rodent model of systemic polymicrobial sepsis”.

Contreras M, Costello J, Higgins B, O’Toole D, Hassett P, Laffey JG.

Western Anaesthesia Symposium, Westport, April 2008

O’Beirne Medal winner

2. “Hypercapnic acidosis attenuates inflammatory response in ventilator induced lung injury by inhibition of the NF-kB pathway”

Contreras M, Curley G, Higgins B, O’Toole D, Ansari B, Laffey JG

11th Current Controversies in Anaesthesia and Peri-operative Medicine Conference. Dingle, October 2009

2nd prize winner
CHAPTER 12 REFERENCES


Villar J, Kacmarek RM, Perez-Mendez L, Aguirre-Jaime A. A high positive end-expiratory pressure, low tidal volume ventilatory strategy improves outcome in


Parker JC, Hernandez LA, Longenecker GL, Peevy K, Johnson W. Lung edema caused by high peak inspiratory pressures in dogs. Role of increased...


West JB. Respiratory physiology - The essentials. 6th ed: Lippincott Williams & Wilkins 2000.


Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2001;**163**:1376-83.


