

# EARLY TREATMENT WITH NEBULISED SALBUTAMOL WORSENS PHYSIOLOGICAL MEASURES AND DOES NOT IMPROVE SURVIVAL FOLLOWING PHOSGENE INDUCED ACUTE LUNG INJURY

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## Abstract

**Objectives:** To examine the effectiveness of nebulised salbutamol in the treatment of phosgene induced acute lung injury.

**Method:** Using previously validated methods, 12 anaesthetised large white pigs were exposed to phosgene (Ct 1978 ± 8 mg min m<sup>-3</sup>), established on mechanical ventilation and randomised to treatment with either nebulised salbutamol (2.5mg per dose) or saline control. Treatments were given 1, 5, 9, 13, 17 and 21 hours following phosgene exposure. The animals were followed to 24 hours following phosgene exposure.

**Results:** Salbutamol treatment had no effect on mortality and had a deleterious effect on arterial oxygenation, shunt fraction and heart rate. There was a reduction in the number of neutrophils from 24.0% ± 4.4 to 12.17% ± 2.1 (p<0.05) in bronchoalveolar lavage, with some small decreases in inflammatory mediators in bronchoalveolar lavage but not in plasma.

**Conclusion:** Nebulised salbutamol treatment following phosgene induced acute lung injury does not improve survival, and worsens various physiological parameters including arterial oxygen partial pressure and shunt fraction. Salbutamol treatment reduces neutrophil influx into the lung. Its sole use following phosgene exposure is not recommended.

## Introduction

Members of the armed forces are at risk of exposure to a wide range of chemicals and environmental materials during war fighting, operations other than war, and whilst providing aid to civil authorities. Military personnel must be properly prepared to operate in an environment where chemical or biological agents may be deployed and, whilst this has been true since the First World War, more recently the threat also exists for the use of such materials in the civilian environment.

Since it is not always possible to possess advance intelligence of the weapons likely to be encountered, appropriate pre-treatments, if available, may not be in place. In the civilian situation, advance warning is even less likely and pre-treatment of civilians would not be possible. It is necessary to develop post-exposure therapeutic strategies to minimise casualty rates and increase survival in individuals poisoned by chemical agents.

Phosgene is an important industrial chemical currently produced in large quantities (approx 2.3 million tonnes per year) as an intermediate in the manufacture of a multitude of materials including dyestuffs, isocyanates, pharmaceuticals and pesticides [1,2]. This current industrial use, and the proven history of phosgene in warfare maintain the potential for accidental or intentional exposure to this chemical [2, 3]. Such a release into a

densely populated urban area would result in mass casualties and in local health care systems being overwhelmed; there is a need for evidence based treatment guidelines to assist in these circumstances. No specific therapies have been identified for treatment of phosgene poisoning.

Studies in laboratory animals have consistently shown acute exposure to phosgene gas to result in pathophysiological changes to the bronchoalveolar regions of the lung, resulting in non-cardiogenic pulmonary oedema due to increased permeability of the alveolar membrane [4-9]. The low solubility and rate of hydrolysis of phosgene in aqueous media favour its penetration into the gas exchange regions and mean it is not significantly scrubbed out in the upper respiratory tract or conducting airways. Once in the alveolar regions it reacts rapidly with nucleophilic constituents, depletes glutathione and transiently destabilises surfactant. Exposure concentrations high enough to elicit an increased alveolar protein concentration may also be associated with surfactant dysfunction, intra-alveolar accumulation of fibrin and collagen, and increased recruitment and activation of inflammatory cells [10].

The clinical effects of human exposure to lethal concentrations of phosgene have been well characterised, and describe an initial asymptomatic period varying from 6-12 hours post exposure, dependent on dose [4]. Fluid from the blood accumulates in the air spaces and impedes vital gas exchange. Later the clinical effects of acute pulmonary oedema become apparent resulting in severe respiratory distress and death from 24 hours post poisoning.

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Models of phosgene poisoning involving isolated lung preparations or small animals enable investigation of the pathophysiology of phosgene exposure and some effects of potential treatments, but their relevance to human exposure and hence patient outcome is not easy to assess. Current management relies on supportive treatment guided by studies primarily conducted in small animals, reflecting major deficiencies in our understanding of the underlying pathology of the injury.

Due to these deficiencies in knowledge, Dstl have developed a terminally anaesthetised large animal model in order to characterise the pathophysiological responses to phosgene exposure and to assess treatment strategies [8]. This model has demonstrated a significant improvement in survival following phosgene induced acute lung injury using intermittent positive pressure ventilation with high positive end expiratory pressure (PEEP) and low tidal volume [11,12]. This treatment, though effective, requires full intensive care facilities and these scarce resources are likely to be overwhelmed following any large scale exposure. There remains a need for simpler less resource intensive interventions such as commercial off the shelf drug treatments [13,14].

The  $\beta$ -agonist, Salbutamol, in addition to acting as a bronchodilator, increases alveolar fluid clearance by up-regulating apical sodium and chloride channels on alveolar type II pneumocytes. It may also reduce alveolar capillary permeability and so reduce alveolar fluid formation [15-17]. In addition to its effects on fluid transfer across the alveolar membrane, it also reduces neutrophil influx into the lung, blocks inflammatory cytokine release and improves lung mechanics following acute lung injury; though the degree of these effects appears to be variable dependent on the models used for investigation and the mode of delivery of the drug [18]. All of these mechanisms are important in phosgene induced acute lung injury, and any amelioration of them may improve survival.

Clinical studies have demonstrated that intravenous Salbutamol improves lung oedema in established human ARDS, though with no effect on survival [19]. Animal models of ARDS have shown that nebulised Salbutamol is also efficacious, with fewer systemic side effects [20]. Human studies in ARDS have shown that there is adequate drug delivery via the nebulised route, and additionally nebulised Salbutamol prevents high altitude pulmonary oedema in individuals at risk [21,22].

The aim of the current study was to investigate, to proof of principle, whether nebulised Salbutamol was effective in treating phosgene induced acute lung injury over the first 24 hours following exposure. Survival to 24 hours was our primary outcome measure.

## Methods

Large white juvenile female pigs (47-55 Kg) (n=12) were obtained from an approved commercial source. All animals were housed in pairs in straw lined pens, and allowed access to food (standard pig diet) and water ad libitum for 5 days, as previously described [8]. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved after ethical review.

## Surgical procedure

Animals were premedicated with midazolam hydrochloride (Hypnovel, Roche Products Ltd., Welwyn Garden City, Hertfordshire) by intramuscular injection (0.6 mg kg<sup>-1</sup>), and anaesthesia induced by inhalation of isoflourane (1-5%) (IsoFlo, Abbott Laboratories, Kent, UK) in 70% oxygen and 30% nitrous oxide. Animals were then intubated using a size 9 cuffed oral endotracheal (ET) tube. Electrocardiogram and pulse oximetry monitoring was commenced. Cervical vessels were surgically exposed and the left and right internal jugular veins and the left

common carotid artery catheterised. The femoral artery was cannulated with a PiCCO (Pulsion Medical Systems AG, Munich, Germany) catheter. Central venous pressure, central venous oxygenation (CeVOX, Pulsion Medical Systems AG, Munich, Germany) and cardiac output (PiCCO, Pulsion Medical Systems AG, Munich, Germany) were measured continuously. All other monitoring devices were attached to a Propaq 106EL monitor (Protocol systems Inc., Beaverton, USA). A Foley Catheter was introduced into the bladder via an open cystostomy. A maintenance infusion of 0.9% sodium chloride and 4% glucose (2.5 ml kg<sup>-1</sup> h<sup>-1</sup>) was delivered to replace insensible losses.

Following surgery, anaesthesia was switched to continuous intravenous comprising ketamine (Ketaset, Fort Dodge Animal Health Ltd, Southampton, Hampshire), midazolam hydrochloride and alfentanil hydrochloride (Rapifen, Janssen Pharmaceuticals Ltd, County Cork, Ireland) as previously described [23]. Animals were then exposed, whilst spontaneously breathing, to phosgene (Ct (product of concentration (mg m<sup>-3</sup>) and time (min)) (Ct 1978  $\pm$  8 mg min m<sup>-3</sup>) as previously described [8]. Between 30 mins and 1 hour following exposure, anaesthesia was deepened and the animals were established on a mechanical ventilator (Evita II, Draeger Ltd) using Intermittent Positive Pressure Ventilation, FiO<sub>2</sub> 0.21, tidal volume 10 ml Kg<sup>-1</sup>, frequency 20 breaths min<sup>-1</sup>, PEEP 3cm water.

## Treatment regimens

All animals were exposed to inhaled phosgene. Animals were then randomly allocated to treatment or control groups, n=6 per group. Treatment consisted of Salbutamol (2.5 mg in 6ml 0.9% saline) administered as an aerosol generated over 20 min by an Aeroneb® Lab micropump nebuliser (Aerogen (Ireland) Ltd, Galway Ireland). Treatments were given at 1, 5, 9, 13, 17 and 21h post phosgene exposure, via the inspiratory cycle of the ventilator. Control group animals were treated with 0.9% saline using the same procedure.

## Measurements

Baseline measurements were taken every 20 minutes for 1 hour pre exposure. Following exposure physiological measurements were recorded every 30 minutes until the end of the experimental period (24h). Additional measurements were also made every 5 mins for 30 mins following each treatment. Derived variables were calculated using standard formulae [24]. Arterial and mixed venous blood samples were taken at hourly intervals and immediately analysed using a GEM blood gas analyser (GEM Premier 5300, Instrumentation Laboratory). Hourly arterial samples were taken into appropriate anticoagulants, centrifuged (3000 rpm, 10 min) and plasma stored at -70°C for subsequent measurement of interleukin (IL) 1, IL-6, matrix metalloproteinase 2 and C-reactive protein using commercial ELISA kits (R&D Systems, Abingdon, Oxon, UK). Haematological analysis was performed on EDTA blood samples using a Coulter Ac-T 5 diff CP (Beckman Coulter) series analyser. Differential white blood cell (WBC) counts were performed manually, following blood smear formation onto a glass microscope slide and staining with DifQuik.

At the conclusion of the 24 hour observation period or when the animal became moribund (defined as asystole and central venous oxygenation of <15%), the animal was culled by an intravenous overdose of sodium pentobarbitone (200mg ml<sup>-1</sup>) (Euthatal, Rhone Merieux Ltd., Harlow, Essex), and a full post mortem examination performed. Following clamping of the trachea distal to the end of the ET tube, the lungs and heart were removed. A flexible bronchoscope (Olympus BF-4B2, KeyMed Ltd, Essex) was inserted into the right median lobe. Bronchoalveolar lavage (BAL) was performed using sterile saline. Aliquots (4 x 40ml) were inserted into the lobe, the fluid being

aspirated between each aliquot and placed on ice. Lavage fluid was analysed for total WBC count (Coulter Ac-T 5 diff CP - Beckman Coulter) and for differential cell count (Shandon Cytospin, 1800 rpm, 10 mins). Slides were stained with DifQuik stain and 100 cells counted. Protein content of BAL supernatant was determined using the Coomassie blue method [25]. Remaining supernatant was stored at  $-70^{\circ}\text{C}$  for subsequent analysis of IL-1, IL-6, IL-8 and tumour necrosis factor using commercial ELISA kits (R&D Systems, Abingdon, Oxon, UK).

Following lavage, the lungs were weighed with the weight of the remaining lavage saline taken into account for lung wet weight / body weight determinations. Samples from each lobe, and all major organs were taken, fixed in neutral buffered formalin and processed for histopathological examination using routine techniques.

### Statistical analysis

Statistical analysis was performed using area under the curve determinations in a 2-tailed Students T test for the following time points: -1 to 0, 0 to 1, 2 to 5, 6 to 9, 10 to 13, 14 to 17, 18 to 21 and 22 to 24h. Results were expressed as mean  $\pm$  standard error (SEM) and p values  $< 0.05$  were considered significant.

## Results

### Mortality

There was no difference in mortality between treatment and control groups, with 4 animals surviving to 24 hours in the control group and 5 animals in the treatment group.

### Oxygenation

Arterial partial pressure of oxygen (PaO<sub>2</sub>) was reduced in the treatment group compared to control throughout the study. This reduction achieved statistical significance ( $p < 0.05$ ) from 5 to 13 and 17 to 21 hours following phosgene exposure (Figure 1).

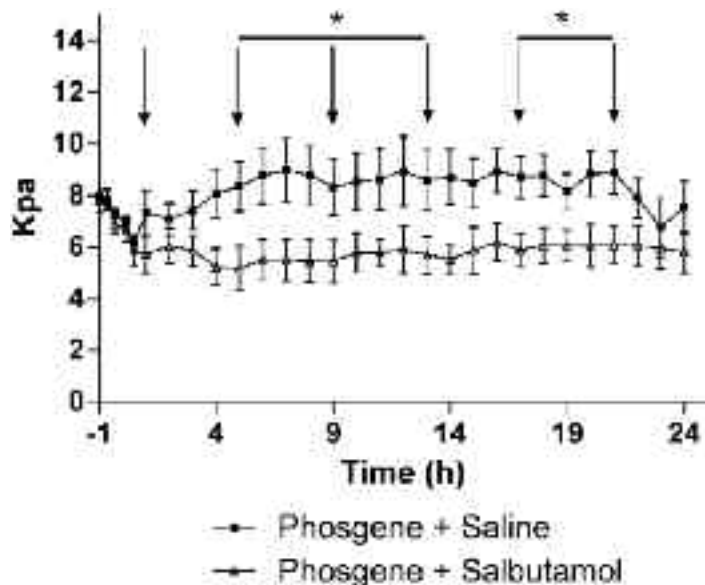


Figure 1. Changes in arterial oxygen partial pressure (PaO<sub>2</sub>) in animals exposed to Phosgene ( $Ct\ 1978 \pm 8\ \text{mg}\ \text{min}\ \text{m}^{-3}$ ) and treated with either Saline (6ml, 0.9%) or Salbutamol (2.5mg in 6ml 0.9% saline) given at 1, 5, 9, 13, 17 and 21h following exposure as indicated by arrows. Salbutamol group had significantly reduced PaO<sub>2</sub> from 5 to 13, and 17 to 21 hours compared to controls ( $p < 0.05$  as indicated by asterisked bars). Values are Mean  $\pm$  SEM,  $n = 6$ .

### Shunt fraction

Shunt fraction ( $Q_s:Q_t$ ) is a calculated measure of the amount of blood passing through the lungs unoxygenated. The Salbutamol group had increased shunt fraction throughout the study compared to untreated controls. This increase was statistically significant from 1 to 9 hours following phosgene exposure ( $p < 0.05$ ) (Figure 2).

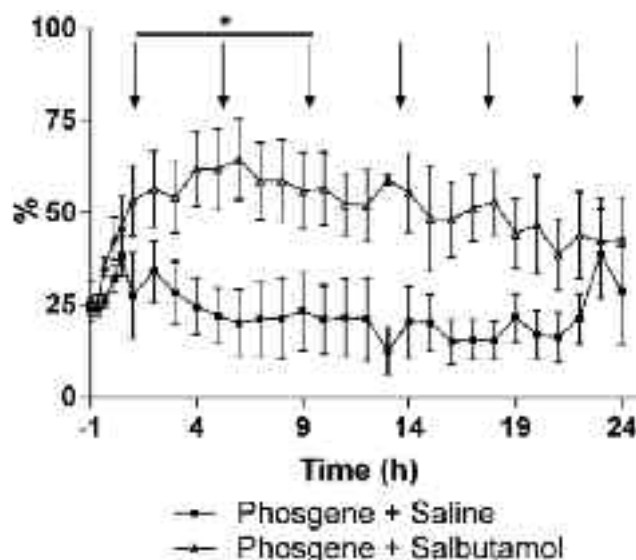


Figure 2. Changes in shunt fraction ( $Q_s:Q_t$ ) in animals exposed to Phosgene ( $Ct\ 1978 \pm 8\ \text{mg}\ \text{min}\ \text{m}^{-3}$ ) and treated with either Saline (6ml, 0.9%) or Salbutamol (2.5mg in 6ml 0.9% saline) given at 1, 5, 9, 13, 17 and 21h following phosgene exposure as indicated by arrows. Salbutamol group had significantly increased shunt fraction from 1 to 9 hours compared to control ( $p < 0.05$  as indicated by asterisked bars). Mean  $\pm$  SEM,  $n = 6$ .

### Heart Rate

Immediately following dosing with Salbutamol, the heart rate increased indicating systemic availability of the drug. The tachycardic response reduced in magnitude following repeated treatments. Heart rate was significantly increased in Salbutamol treated animals compared with controls from 5 to 13 hours following phosgene exposure ( $p < 0.0001$ ). No significant difference in heart rate was detected after 13 hours (Figure 3).

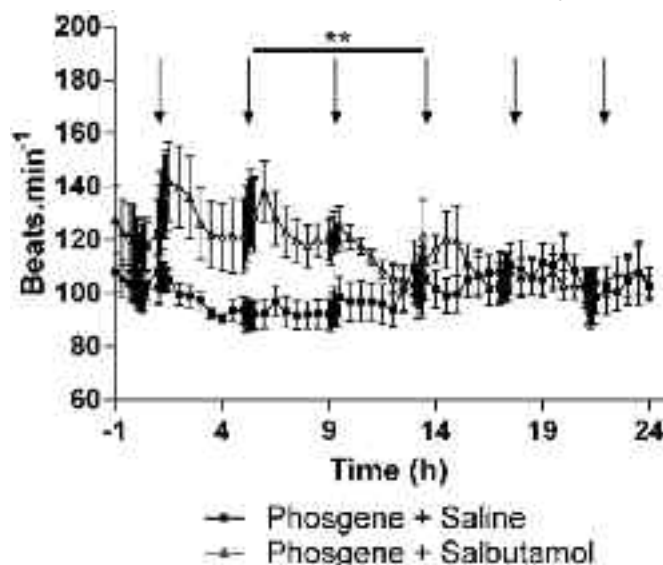


Figure 3. Changes in heart rate in animals exposed to Phosgene ( $Ct\ 1978 \pm 8\ \text{mg}\ \text{min}\ \text{m}^{-3}$ ) and treated with either Saline (6ml, 0.9%) or Salbutamol (2.5mg in 6ml 0.9% saline) given at 1, 5, 9, 13, 17 and 21h following phosgene exposure as indicated by arrows. Salbutamol group had significantly increased heart rate from 5 to 13 hours compared to controls ( $p < 0.0001$  as indicated by asterisked bars). Mean  $\pm$  SEM,  $n = 6$ .

### Bronchoalveolar lavage

Differential WBC count on BAL fluid from animals not exposed to phosgene shows that neutrophils make up  $< 5\%$  of the total normal cell population of the lung (historical data not shown). Following phosgene exposure, there was a significant increase in the proportion of neutrophils compared with unexposed historical controls (24 %  $\pm$  4.4;  $p < 0.05$ ). Treatment with inhaled Salbutamol resulted in a significant reduction in neutrophil sequestration into the lung following exposure to phosgene (12.17 %  $\pm$  2.1;  $p < 0.05$ ) (Figure 4).

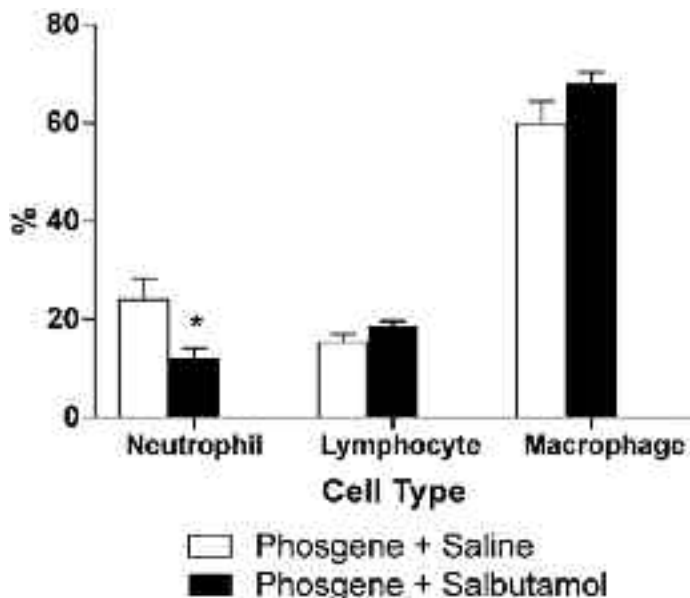


Figure 4. Differential white blood cell count from terminal bronchoalveolar lavage in animals exposed to Phosgene ( $Ct 1978 \pm 8 \text{ mg min m}^{-3}$ ) and treated with either Saline (6ml, 0.9%) or Salbutamol (2.5mg in 6ml 0.9% saline) given at 1, 5, 9, 13, 17 and 21h following phosgene exposure. Salbutamol group had significantly decreased number of neutrophils compared to controls ( $p < 0.05$  as indicated by asterisk). Mean  $\pm$  SEM,  $n = 6$ .

### Additional Parameters

There were no significant differences in cardiac output, mean arterial pressure, extravascular lung water, lung wet weight to body weight ratio, inflammatory mediators or peripheral WBC count between treatment and control groups (data not shown).

### Discussion

This study has shown that repeated nebulised Salbutamol treatment of phosgene induced acute lung injury reduced neutrophil influx into the lung, did not affect survival, but worsened several physiological parameters including arterial oxygenation and shunt fraction.

Previous human studies using intravenous Salbutamol in established ARDS (the BALTI trial) have shown a reduction in extravascular lung water (EVLW), which was not seen in our data [19]. Although both studies used Salbutamol in an attempt to ameliorate pathophysiological changes following acute lung injury, there are important differences in patient population, lung injury, drug delivery and timescale. Our study used a porcine model of acute lung injury in order to examine an injury that cannot be studied in humans. Although pig models of acute lung injury are well established and widely accepted to reflect accurately the changes seen in human lung injury, it is possible, though unlikely, that the difference in results is due to inter-species differences [26,27,28]. It is more likely however, that differences in injury or drug delivery are pivotal factors. The BALTI study examined patients within 48 hours of onset of ARDS when already established on mechanical ventilation, whereas our study examined the effect of Salbutamol immediately following an acute lung injury. ARDS and phosgene induced lung injury share many common elements, but the patients in the BALTI trial had relatively stable, established disease, whereas in our model the injury was acute and dynamic. The effects of Salbutamol in rapidly developing pulmonary oedema may be different from that where the oedema is more stable, and although Salbutamol has been shown to prevent the rapid formation of high altitude pulmonary oedema, this is of a different magnitude of severity, and was examined using a pre-treatment strategy [22].

We examined Salbutamol via the inhaled route in order to mimic drug delivery that is most readily available following a large-scale phosgene release. The BALTI trial used intravenous

Salbutamol, though other studies have demonstrated that drug delivery via the nebulised route is equally efficacious [21]. Though we demonstrated no improvement in survival or positive changes in physiological parameters, the changes that we did observe indicate there was effective drug delivery inducing measurable physiological change. In our acute model of lung injury the formation of alveolar fluid is rapid and overwhelms mechanisms in place for its removal. The addition of Salbutamol may improve clearance, but to such a small degree that it is below the level of our detection in the first 24 hours following injury, or the level of injury is so severe that it completely halts the active metabolic reabsorption of alveolar fluid. In ARDS the production of alveolar fluid is elevated, but relatively stable; the addition of Salbutamol and a consequent rise in alveolar fluid clearance is sufficient to result in clinical improvement, but even in this situation requires several days; improvements in EVLW in the BALTI trial were only seen after 4 days of treatment.

In keeping with our policy of refinement, reduction and replacement where possible of laboratory animal use, the study was performed with low experimental numbers. Such small numbers lead to reduced sensitivity of the experimental series, with potential for false negative results. Despite the low experimental numbers some statistically significant differences were observed.

Arterial oxygenation and shunt fraction worsened in the Salbutamol treatment group in keeping with a worsening of ventilation / perfusion matching. In an ideal lung, ventilation to any individual alveolus would be matched by its blood flow or perfusion. More precisely, alveolar gas exchange is optimal in alveoli when the fraction of alveolar ventilation (VA) is matched to the fraction of cardiac output (Q) to that specific alveolus. This ideal situation does not occur throughout the normal lung, with ventilation / perfusion (V/Q) matching varying from the base to apex of the lungs due to variations in blood flow and ventilation mostly due to gravity in the upright lung. Salbutamol is removed from the lung by blood flow and is primarily renally excreted. Areas of the lung with poor perfusion clear the administered Salbutamol more slowly than areas with good blood supply; this enables greater bronchodilation in the areas of the lung with poorer blood supply and vice versa, hence worsening V/Q matching.

Heart rate increased in the Salbutamol treated animals when compared to the saline treated controls. This was particularly noticeable immediately following Salbutamol treatments given at early time points demonstrating that the Salbutamol was effectively delivered and absorbed systemically. The accommodation in the cardiac response is expected and seen in human asthmatics given repeated Salbutamol doses [29].

In our model, phosgene inhalation resulted in neutrophil influx into the lungs; ARDS is also characterised by intense inflammation in the alveolar space, dominated by neutrophils. We have shown a 50% reduction in bronchoalveolar lavage neutrophils following repeated nebulised Salbutamol, however following intravenous Salbutamol there was no change in the proportion of BAL neutrophils in human ARDS [30]. This may be due, as discussed above, to the different mode of drug delivery, though experiments carried out on sheep examining the effects of  $\beta$ -agonists on endothelial and epithelial leakage demonstrate no difference in the two modes of drug delivery [31]. Therefore it is more likely that the difference seen is due to the acute response in our model compared to the relatively stable injury pattern in ARDS. In the human ARDS patients the neutrophil population may be fairly static, hence no changes were seen in BAL neutrophil count. In our model there is a large influx of neutrophils which is reduced by Salbutamol treatment. It has previously been shown that inhaled  $\beta$ -agonists inhibit acute lipopolysaccharide (LPS) induced neutrophil influx in human volunteers, and reduce neutrophil chemotaxis in vitro [30,32].

In studying the effects of acute lung injury induced by phosgene in any model there are inherent restrictions. The current study only examined clinical and physiological parameters to 24 hours after phosgene exposure; as such delayed deterioration or improvement in the treatment group after this time could not be assessed, but would be of significant clinical interest. The reduction of lung neutrophils may have gone on to reduce the overall severity of the lung injury had the model been followed over a number of days.

The use of inhaled Salbutamol alone as a treatment in the early stages (up to 24 hours) of phosgene induced acute lung injury is not recommended.

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