A novel two-hit rodent model of postoperative acute lung injury: priming the immune system leads to an exaggerated injury after pneumonectomy†

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Abstract

OBJECTIVES: Postoperative acute lung injury (PALI) is a rare, poorly understood, usually fatal condition, accounting for the majority of deaths following lung resection. Its low frequency and unpredictable development make the identification of the mechanisms of injury from clinical studies alone almost impossible. Multiple validated ‘two-hit models’ exist for ALI secondary to other causes. We describe a novel rodent ‘two-hit’ model of PALI: a low-grade immune stimulus, such as sepsis, greatly aggravates the injury in the remaining lung observed following pneumonectomy.

METHODS: Under general anaesthesia, rats received either low-dose intratracheal lipopolysaccharide (IT-LPS) challenge (10 µg for 1 h) followed by left posterolateral thoracotomy, one-lung ventilation (OLV), pneumonectomy and 3 h of ventilation; 500 µl IT 0.9% saline followed by the same surgery or IT-LPS followed by sham surgery and ventilation. All other conditions were constant. Lung injury is heralded by neutrophil accumulation, which was determined by right lung bronchoalveolar lavage cell count. Data are presented as mean ± standard error of the mean. The T-test was used to compare normally distributed groups with correction for multiple comparisons.

RESULTS: A dose–response curve identified the clinically relevant ‘low dose’ of LPS to be used in further studies. Ventilatory parameters were standardized to reflect clinical practice (volume-control, tidal volume of 6 ml/kg, positive end-expiratory pressure of 2 cmH2O, maximum airway pressure of <15 cmH2O). There was a degree of adaptation to obtain a consistent and robust model with retest validity. OLV and pneumonectomy alone produced a small lung injury (65.1 ± 5), as did 10 µg intratracheal LPS alone (50.7 ± 6.9). However, when OLV, pneumonectomy and 10 µg LPS were combined, an exaggerated injury occurred (161.4 ± 10.3), P = 0.007.

CONCLUSIONS: Early results show that a two-hit model of PALI is viable and that sepsis aggravates the response to pneumonectomy. The model is now being further characterized. Once established, this model will offer the chance to better understand PALI and to develop and test novel therapies and risk reduction strategies for the condition.

Keywords: Postoperative acute lung injury • Acute respiratory distress syndrome • Pneumonectomy • Lung resection • Surgical model • Lobectomy

INTRODUCTION

Postoperative acute lung injury/acute respiratory distress syndrome (PALI/ARDS) is a well-recognized complication following major lung resection surgery with an incidence of up to 7% [1]. The condition was previously known as ‘post-pneumonectomy pulmonary oedema’ or ‘non-cardiogenic pulmonary oedema’. American-European Consensus Diagnostic Criteria for PALI/ARDS are bilateral pulmonary infiltrates on chest radiograph, acute onset, normal cardiac filling pressures (pulmonary artery wedge pressure <18 mmHg) and, additionally, a PaO2:FiO2 ratio <300 mmHg in PALI and <200 mmHg in ARDS [2]. PALI and ARDS represent separate ranges within the spectrum of lung injury severity. PALI/ARDS is frequently fatal (>50%) [1] and, even in the outcome of survival, the morbidity resulting from a prolonged period of ventilation often results in a significant reduction in the quality of life; therefore, the development of novel risk reduction and treatment strategies is highly desirable. Major lung resections are principally performed for the treatment of primary lung cancer and secondary extrapulmonary metastatic cancer; therefore, improvement in the survival of PALI/ARDS

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will result in improved survival for these conditions. The rarity and unpredictability of PALI/ARDS make the study of the mechanisms of injury clinically almost impossible; therefore, an in vivo mammalian surgical model of post-pneumonectomy PALI/ARDS is required to advance our understanding of its pathogenesis.

The delay in the presentation of PALI/ARDS days after initial surgery suggests that a two-hit or multiple-hit aetiology is likely. We postulated a two-hit hypothesis; preoperative low-grade lung sepsis and OLV in combination will cause a significant lung injury. Moreover, we hypothesized that the combination of low-grade lung sepsis and OLV would synergise and cause a greater lung injury than either effect in isolation. The primary aim of our model was thus to assess this hypothesis and to develop a reliable and reproducible surgical model protocol for PALI/ARDS.

MATERIALS AND METHODS

Male Wistar rats fed on standard rat chow weighing 300–350 g were used in all experiments. All work was carried out under a UK Home Office Project Licence under the Animals (Scientific Procedures) Act 1986 with full approval from the University of Warwick Bioethics Committee. Our model of lung sepsis was to use intratracheal instillation of lipopolysaccharide (IT-LPS; 10 µg in 500 µl normal saline). LPS serotype 0111: B4 (Sigma-Aldrich, USA) was used for all experiments. The use of this serotype of IT-LPS as a model of acute lung injury in the rat has been validated and reported by several groups using this serotype [3, 4]. LPS is a component of the cell wall of gram-negative bacteria that is commonly known as endotoxin and is commonly used to model sepsis in vivo [5]. General anaesthesia was induced and maintained using inhalational 2% isoflurane, atroventin 6 µg was administered and 1 ml of normal saline was administered at hourly intervals to all animals. Following induction, the trachea was exposed and IT-LPS was injected into the bronchoalveolar tree. Rectal temperature was monitored and maintained at 38–39°C.

An LPS dose–response curve series was performed using 5 h of self-ventilation with a face mask under general anaesthesia. At 5 h, terminal anaesthesia was induced, the animals were exsanguinated by an incision in the inferior vena cava and the heart-lung blocks were harvested. Bronchoalveolar lavage (BAL) using 6 ml of normal saline at 4°C was used to retrieve alveolar cellular infiltrates (predominantly neutrophils). BAL neutrophil cell counts were performed using a haemocytometer as an index of the severity of acute lung injury. Whole-lung neutrophil counts were corrected by the formula: whole-lung BAL neutrophil cell count/volume of fluid retained (ml) = standardized neutrophil cell count.

The surgical model was carried out using an SAR-830 small animal ventilator (Linton Instrumentation, UK) integrated into a circuit that included an inflatable oxygen reservoir. Using oxygen flows of 600 ml/min, respiratory rate of 60 breaths per minute, an inspiratory:expiratory (I:E) ratio of 1:1, a tidal volume of 2.5 ml and fraction of inspired oxygen concentration (FiO₂) of 60%, positive end-expiratory pressure of 2 cmH₂O, maximal circuit pressures of 20 cmH₂O and 2% isoflurane inhalational anaesthesia, animals were ventilated. IT-LPS/sham saline was administered followed by 1 h of self-ventilation via a face mask. At 1 h, a tracheostomy was fashioned, a 14-gauge endotracheal cannula was placed and positive-pressure ventilation was commenced at the above settings via the endotracheal cannula. Adequacy of ventilation was assessed by the absence of spontaneous respiratory effort.

The surgical model was performed in four variations or groups (Table 1) concerning 10 µg IT-LPS vs sham IT-LPS (saline), OLV vs double-lung ventilation (DLV) and thoracotomy + pneumonectomy vs sham thoracotomy. All other conditions were constant in all experiments. In the early stages, a degree of adaptation was used to obtain a robust and reliable model. The posterolateral thoracotomy was performed in the fifth intercostal space. Ten microgram of LPS was chosen for the surgical model as a dose that caused a small but significant lung injury. Following thoracotomies, the lungs of all animals were kept moist with the periodic application of warm normal saline and covering the incision with a plastic wrap.

OLV was induced by advancing the endotracheal cannula snugly into the right main bronchus. OLV was confirmed by ensuing atelectasis of the left lung in the absence of spontaneous respiration with a beating heart, indicating adequate ventilation. It was occasionally necessary to secure the endotracheal cannula with a ligature to maintain OLV with a complete seal. At pneumonectomy, the pulmonary hilar vessels (pulmonary arteries and pulmonary veins) were double-liga-clipped en masse along with the main bronchi. The stump was then divided distal to the clips and the left lung removed. In the sham thoracotomies, the thorax was opened; however, no pneumonectomy was performed. Recovery from general anaesthesia under the terms of our UK Home Office Project Licence was prohibited. Therefore, at 5 h, terminal anaesthesia was induced in all animals using 5% isoflurane. A midline laparotomy was performed and 5 ml of blood was drawn from the inferior vena cava of all animals for the future assessment of serum markers. Following aspiration, the inferior vena cava was incised and the animal was exsanguinated. A median sternotomy was performed and the heart-lung blocks were harvested.

For all surgical model experiments, right lung BAL was performed using 3 ml of cold normal saline with three cycles of instillation/aspiration. Where both lungs remained (i.e. in Groups 3 and 4), the left lung hilum was double-liga-clipped so that only the right lung was lavaged. BAL cell counts were performed. Data are presented as mean ± standard error of the mean (SEM). The T-test was used to compare normally distributed groups with correction for multiple comparisons.

RESULTS

The IT-LPS dose–response series of experiments showed the BAL neutrophil cell count to rise in a predictable fashion as the LPS dose increased (Fig. 1; 0 µg LPS: n = 4; 10 µg LPS: n = 4; 100 µg LPS: n = 2; 250 µg LPS: n = 2; 500 µg LPS: n = 2). A dose of 10 µg LPS was chosen for the surgical model as it just produced a measurable rise in lavage cell counts increasing from 14.2 ± 10.5 neutrophils/ml ± 10.5 in the control group to 68 ± 5.3 in the 10 µg IT-LPS group (P < 0.001). At doses of 250–500 µg, massive undifferentiated lung injuries occurred; therefore, experiments were not repeated more than twice. In a series of 16 animals, all undergoing and surviving the surgical protocol (four in each group), BAL neutrophil cell counts confirmed the two-hit hypothesis; OLV and pneumonectomy alone (Group 2) resulted in a small lung injury (65.1 ± 5), as did 10 µg IT-LPS alone (Group 3) (50.7 ± 6.9). However, when OLV, pneumonectomy and 10 µg LPS were combined in the same animal (Group 1), an
exaggerated injury occurred (161.4 ± 10.3). This represented a magnitude of lung injury significantly greater than the sum of the two effects seen in Groups 2 and 3 (P = 0.007 and 0.004). T-test comparing Groups 1 and 2. T-test comparing Groups 1 and 3.

DISCUSSION

The preliminary results of this study demonstrate its technical feasibility and provide early validation of this two-hit rodent model of PALI after lung resection. This is the first study to demonstrate the synergistic deleterious effects of low-dose immune stimulation and surgery (OLV and lung resection); in combination, these effects were associated with a 2.8-fold increase in the severity of lung injury compared with their effect alone. These results are exciting, however, at this stage further studies are needed to validate the model, namely in the other major indices of lung injury, e.g. endothelial permeability, and to define the cascade of immune activation postoperatively. PALI/ARDS presents at variable postoperative intervals ranging from shortly after surgery to several days. It would be desirable to allow full postoperative recovery from anaesthesia to better mimic the clinical situation and to assay acute lung injury in the postoperative period for a number of days, however, to minimize suffering, terminal anaesthesia was induced at the end of surgery. The model, however, is still valid as a means of assessing a two-hit hypothesis in the subset of patients who rapidly develop PALI/ARDS in the postoperative period (in our model surgery is complete at 2 h and a further period of ventilation only ensues for 3 h). In addition, our results indicate that the immune system is primed within this short-time interval. Aerosolized LPS is a viable alternative to the instillation of LPS in saline solution; however, both methods provide good coating of the inner surface of the bronchoalveolar tree.

### Table 1: Surgical model protocol

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group 1 (LPS + OLV + pneumonectomy)</th>
<th>Group 2 (sham LPS + OLV + pneumonectomy)</th>
<th>Group 3 (LPS + DLV + sham surgery)</th>
<th>Group 4 (sham LPS + DLV + sham surgery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µg IT-LPS</td>
<td>Sham IT-LPS (normal saline)</td>
<td>10 µg IT-LPS</td>
<td>Sham IT-LPS (normal saline)</td>
</tr>
<tr>
<td>1</td>
<td>Endotracheal intubation and positive-pressure ventilation</td>
<td>Endotracheal intubation and positive-pressure ventilation</td>
<td>Endotracheal intubation and positive-pressure ventilation</td>
<td>Endotracheal intubation and positive-pressure ventilation</td>
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<tr>
<td></td>
<td>Left posterolateral thoracotomy</td>
<td>Left posterolateral thoracotomy</td>
<td>Left posterolateral thoracotomy</td>
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<tr>
<td>2</td>
<td>Induction of OLV (1 h)</td>
<td>Induction of OLV (1 h)</td>
<td>Induction of OLV (1 h)</td>
<td>Induction of OLV (1 h)</td>
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<tr>
<td></td>
<td>Left pneumonectomy</td>
<td>Left pneumonectomy</td>
<td>Left pneumonectomy</td>
<td>Left pneumonectomy</td>
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<tr>
<td>5</td>
<td>Terminal anaesthesia</td>
<td>Terminal anaesthesia</td>
<td>Terminal anaesthesia</td>
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<tr>
<td></td>
<td>5 ml whole blood aspirated and stored</td>
<td>5 ml whole blood aspirated and stored</td>
<td>5 ml whole blood aspirated and stored</td>
<td>5 ml whole blood aspirated and stored</td>
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<td></td>
<td>Exsanguination</td>
<td>Exsanguination</td>
<td>Exsanguination</td>
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<td></td>
<td>Heart-lung block harvest</td>
<td>Heart-lung block harvest</td>
<td>Heart-lung block harvest</td>
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<tr>
<td></td>
<td>Bronchoalveolar lavage/permeability index assay</td>
<td>Bronchoalveolar lavage/permeability index assay</td>
<td>Bronchoalveolar lavage/permeability index assay</td>
<td>Bronchoalveolar lavage/permeability index assay</td>
</tr>
</tbody>
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LPS: lipopolysaccharide; OLV: one-lung ventilation; DLV: double-lung ventilation; IT: intratracheal.
Major lung resection creates unique intraoperative and post-operative pathophysiological circumstances that are thought to promote the development of PALI/ARDS [6]. Intraoperatively, a period of OLV with atelectasis of the operative lung is necessary to permit dissection and ligation of major blood vessels and airways. Cessation of ventilation of the operative lung achieved via insertion of auffed double-lumen endobronchial tube in the contralateral main bronchus results in atelectasis of the operative lung with consequent cellular hypoxia within the lung parenchyma due to the lack of ventilation and reflex hypoxic pulmonary vasocostriction [7]. Brief episodes (~60 min) of atelectasis (which are inevitable in OLV) have been shown to activate lung inflammatory cells, such as the alveolar macrophage, and allow them to produce proinflammatory cytokines [8]. But OLV alone is not sufficient to induce significant acute lung injury. This is consistent with the clinical finding of a very low incidence of PALI in patients undergoing OLV for non-lung resection procedures, e.g. pleurectomy/decostization. Surgical handling of tissues may initiate and/or aggravate lung injury, particularly in the case of major blood vessels; this is supported by the higher incidence of PALI with more complex surgery [9]. During OLV, reflex hypoxic pulmonary vasocostriction reduces the ventilation-perfusion mismatch, further compounding the degree of hypoxia within the lung parenchyma (however, blood flow to the ventilated lung is accentuated, aiding in systemic oxygenation). Simultaneously, the ventilatory parameters required to maintain adequate oxygenation and ventilation via the isolated contralateral lung also have directly injurious effects. High inspiratory pressures cause barotrauma and volutrauma. Discontinuation of OLV in sub-total lung resections (e.g. lobectomies) has been shown to paradoxically result in further cellular damage; following re-expansion, reoxygenation and reversal of hypoxic pulmonary vasocostriction result in an ischaemia-reperfusion injury to the remaining lung [10]. The ischaemia-reperfusion sequence results in massive generation of highly reactive oxygen-free radicals, overwhelming the normal defences against oxidative stress and resulting in endothelial cell dysfunction [10]. All evidence suggests that the pathogenesis of PALI/ARDS is almost certainly highly multifactorial with no single identified event sufficient to cause the phenomenon alone. Dissection of the individual contribution of each of these described components is the next stage in the development of this model and will give us a better understanding of the mechanisms of the development of PALI.

We believe that the surgical stimulus may result in remote immune activation of the remaining lung tissue, generating a proinflammatory response postoperatively, priming it to an exaggerated response to a second ‘hit’, e.g. postoperative chest sepsis. Immune activation of surgery [11, 12] likely predisposes to the development of PALI, because suppression of the immune system is associated with a reduction in the inflammatory response following surgery, and suppression intraoperatively with a single dose of steroid is associated with a reduction in the incidence of PALI [13].

Clinical trials in PALI on these questions are inherently difficult to conduct; PALI/ARDS development is unpredictable and cases are small in number (due to its occurrence in <5% of all lung resection cases). It is not possible to accurately assess the individual contributions of OLV and surgery in human subjects, because there is no access to tissue from the remaining lung, moreover, clinical studies of this type could raise serious ethical issues. Therefore, in order to make therapeutic and/or preventative advances, our understanding of this condition must be enhanced by in vivo surgical models. Multiple validated models of lung resection surgery already exist, beginning with the 1984 canine study demonstrating excessive fluid administration to cause post-resectional pulmonary oedema [14]. In addition, multiple validated two-hit models of PALI/ARDS exist in the literature in varied species (rat, mouse, rabbit and pig) [15]. In these models, LPS is the ‘first hit’ and has been delivered via the intratracheal, intraperitoneal, intravenous and aerosolized route. Second hits described in these models include hypoxia, haemorrhage, acid aspiration and ischaemia-reperfusion injury. These models all strengthen the evidence for the multifactorial aetiology of PALI. Despite this wealth of two-hit models for ALI/ARDS, no published studies currently provide a model of OLV, immune activation and lung resection as described here in our novel rat model. This species was selected for technical ease of OLV, for the strong similarities between rodent and human respiratory and immune physiology, and the knowledge that rat models of surgery have successfully been translated into clinical practice without the need to repeat the model in other larger species. Evidence for the two-hit model has been found in retrospective observational clinical studies and second hits, including excessive intraoperative blood loss associated with hypotension, massive blood transfusion, excessive IV fluid administration in the peri-operative period, long periods of OLV and ventilator-induced lung injury (as a result of volutrauma/barotrauma), have been identified [16].

Our rodent surgical model will need to be linked to clinical data to allow validation. For example, in a clinical study comparing a protective lung ventilation (PLV) protocol (n = 558) with standard lung ventilation (n = 533), PLV (small-tidal volume, limiting maximal pressure ventilation and adding end-expiratory positive pressure along with alveolar recruitment manoeuvres) was shown to be associated with a reduction in the incidence of PALI of 0.9 vs 3.7% [17]. These types of protective strategies could first be tested and optimized within a rodent model prior to conducting larger clinical trials. In addition, the key to the success of clinical trials will be identifying surrogate markers of injury that can be measured in a clinical trial. The identification of such serum markers could result from this rodent model. In summary, PALI/ARDS following lung resection is a serious and difficult-to-prevent condition with a highly multifactorial aetiology. A novel in vivo surgical model such as this is an essential tool for identifying novel treatments and preventive strategies to ultimately reduce the incidence, morbidity and mortality of this condition.

Conflict of interest: none declared.

REFERENCES

APPENDIX. CONFERENCE DISCUSSION

Dr C. Choong (Melbourne, Australia): My first question is in two parts. Part A: How many rats were utilized to figure out the approximate dose response results which finally led to choosing 10 μg LPS as a dose of choice? This was not stated in your manuscript or your presentation.

Dr Evans: There were dosage groups of 10, 50 to 100 micrograms; there were four animals in each of these groups. For the larger doses we only performed experiments once or twice because there was a massive injury that wasn’t differentiable.

Dr Choong: And Part B: You have four groups and in each group there were four rats. How did you come to this number of rats in each group and do you think that is sufficient?

Dr Evans: My supervisor carried out powering studies to determine that n=4 would be sufficient to show statistical significance in this event. We found that that was sufficient to show a highly statistically significant result. And obviously, with the principles of reducing suffering to the animals, we needed to use the least number of animals as possible.

Dr Choong: Secondly, you have very nicely explained in your manuscript why you have chosen the rodent model. You have carefully explained the various contributing factors of acute lung injury in the clinical real-life situation of single-lung ventilation and pulmonary resection surgery in both the discussion part of your manuscript and in your slide presentation today. You have carefully designed your experimental study to look at these factors.

For my second question was, I was going to ask you about where you and your coauthors were going to go from here. However, I think you’ve very nicely explained that in the last part of your presentation.

Dr N. Cartwright (Hull, UK): I think it’s fantastic, lovely, clear data which shows synergy between the mock operation and LPS. Have you thought about what the underlying signalling mechanism is behind this synergy and how you could show that to be the case? Did you use ultrapure LPS or stunted LPS, and have you thought about how you may show its synergy in a TLR signalling pathway?

Dr Evans: As in toll-like receptor signalling?

Dr Cartwright: Yes.

Dr Evans: We used an endotoxin from Sigma, a serotype, which had been used in several previous two-hit models which we looked up in our literature review. In terms of the mechanism of the synergy, that remains unknown and it is something which we are interested in trying to elucidate in the future by looking at the expression of inflammatory markers, trying to track them at various time points, and following all the insults given during the surgery. And that’s what we’d like to do clinically with blood sampling as well, and at various time points during a pneumonectomy, to try and identify this mechanism.

Dr H. Ankersmit (Vienna, Austria): In your clinical scenario, if you do a pneumonectomy, what is the treatment of choice now? Do you give antibiotics? Because you show surgery plus saline and plus LPS aggravates.

Dr Evans: No, I don’t believe so, other than the prophylactic antibiotics prior to the anaesthetic induction.

Dr P.B. Rajesh (Birmingham, UK): We give one short intravenous antibiotic at the time of induction of anesthesia.

Dr Ankersmit: Just once?

Dr Rajesh: Yes.

Dr Ankersmit: In Vienna, we do it for 5-6 days. And our rationale for this (because we have also done some similar studies) is that we don’t wait for the insult, for the germ to proliferate.

Dr R. Schmid (Bern, Switzerland): We also give prolonged antibiotics in these situations. This is an example where we have a clinical situation, pneumonectomy, where mostly mortality is very low, but a situation where everybody has mortality. And you looked at this clinical situation, based your research around it, and found out the reasons.