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Early Identification of Acute Lung Injury in a Porcine Model of Hemorrhagic Shock

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14. ABSTRACT

Acute lung injury (ALI) is a frequent complication after severe trauma. Lung-protective ventilation strategies have been proposed for the prevention of ALI, however, the efficacy of these strategies on reducing pulmonary inflammation is unclear. We compared lung-protective ventilation to traditional ventilation techniques in a porcine model of hemorrhagic shock and resuscitation. Pigs were randomized to mechanical ventilation with low tidal volume (TV) (6 mL/kg) or high TV (12 mL/kg). After equilibration, animals underwent pressure-controlled hemorrhage (mean arterial pressure 35±5 mmHg) for 1h, followed by resuscitation with shed fresh whole blood or Hextend and were then maintained at mean arterial pressure of 50±5 mmHg for 3h in the post-resuscitation phase. Bronchoalveolar lavage fluids were collected hourly and analyzed for inflammatory markers and lung samples were analyzed with histology and immunohistochemistry after euthanasia. Sham animals were used as negative controls. Pigs that underwent hemorrhagic shock had higher heart rates, lower cardiac output, lower mean arterial pressures and worse acidosis compared to sham animals at the early time points ($p<0.05$ each). There were no significant differences in central venous pressure or pulmonary capillary wedge pressure between groups. Pulmonary neutrophil infiltration, as defined by Ly6G staining on lung samples, was greater in the shock groups regardless of resuscitation fluid ($p<0.05$ each). Serum porcine surfactant protein D levels were not different between groups. Fresh whole blood resuscitation induced increased pulmonary neutrophil accumulation compared to sham in both high and low TV groups, and compared to Hextend in the high TV groups. Overall, low TV did not demonstrate acute pulmonary benefits compared to high TV, but induced increased neutrophil accumulation in the Hextend resuscitation groups only. Our study demonstrates the reproducibility of a porcine model of hemorrhagic shock that is consistent with physiologic changes in humans in hemorrhagic shock. Early implementation of low TV ventilation did not demonstrate any pulmonary benefits. Pulmonary neutrophil infiltration may serve as an early marker for ALI, and may be present prior to clinical changes in pulmonary physiology after hemorrhagic shock.

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1.0 SUMMARY

Acute lung injury (ALI) is a frequent complication after severe trauma. Lung-protective ventilation strategies have been proposed for the prevention of ALI, however, the efficacy of these strategies on reducing pulmonary inflammation is unclear. We compared lung-protective ventilation to traditional ventilation techniques in a porcine model of hemorrhagic shock and resuscitation.

Pigs were randomized to mechanical ventilation with low tidal volume (TV) (6 mL/kg) or high TV (12 mL/kg). After equilibration, animals underwent pressure-controlled hemorrhage (mean arterial pressure [MAP] 35±5 mmHg) for 1h, followed by resuscitation with shed fresh whole blood or Hextend and were then maintained at MAP of 50±5 mmHg for 3h in the post-resuscitation phase. Bronchoalveolar lavage fluids were collected hourly and analyzed for inflammatory markers and lung samples were analyzed with histology and immunohistochemistry after euthanasia. Sham animals were used as negative controls.

Pigs that underwent hemorrhagic shock had higher heart rates, lower cardiac output, lower MAPs and worse acidosis compared to sham animals at the early time points ($p<0.05$ each). There were no significant differences in central venous pressure or pulmonary capillary wedge pressure between groups. Pulmonary neutrophil infiltration, as defined by Ly6G staining on lung samples, was greater in the shock groups regardless of resuscitation fluid ($p<0.05$ each). Serum porcine surfactant protein D levels were not different between groups. Fresh whole blood resuscitation induced increased pulmonary neutrophil accumulation compared to sham in both high and low TV groups, and compared to Hextend in the high TV groups. Overall, low TV did not demonstrate acute pulmonary benefits compared to high TV, but induced increased neutrophil accumulation in the Hextend resuscitation groups only.

Our study demonstrates the reproducibility of a porcine model of hemorrhagic shock that is consistent with physiologic changes in humans in hemorrhagic shock. Early implementation of low TV ventilation did not demonstrate any pulmonary benefits. Pulmonary neutrophil infiltration may serve as an early marker for ALI, and may be present prior to clinical changes in pulmonary physiology after hemorrhagic shock.

2.0 INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common complications after severe trauma [1, 2]. Among survivors requiring large-volume blood transfusion, over 30% develop clinically significant ALI within the first 48 hours [3]. The insults of ALI extend well beyond pulmonary compliance issues, and frequently compromise patient-related outcomes. Nearly 30% of patients who develop trauma-associated ALI succumb to early mortality [4-6]. These patients often require prolonged mechanical ventilation, resulting in ventilator-associated pneumonia rates as high as 50%, and a median hospital stay exceeding one month [7]. Due to challenges in early diagnosis of ALI, however, treatment strategies are often implemented too late to improve patient outcomes. Therefore, methods for early detection and prevention of ALI are imperative to improving care among our injured population.

Currently, two treatment strategies have been proposed for the treatment of ALI – damage control resuscitation and lung-protective ventilation. Damage control resuscitation addresses traumatic hemorrhage and coagulopathy using a balanced blood product resuscitation, while limiting exposure to large-volume crystalloid fluids [8]. Lung-protective ventilation

techniques, through the use of lower tidal volumes (TV), can also prevent further iatrogenic harm to compromised lungs, including barotrauma, volume trauma, and mechanical shear stress [9]. Both strategies have been suggested within the Joint Trauma System and Critical Care Air Transport Team clinical practice guidelines [10, 11]. Furthermore, strong evidence exists to support the use of lower TV for the treatment of ALI and ARDS in the general population [12] The implementation of these strategies in the early treatment of trauma patients, however, and the ultimate goal of preventing trauma-associated ALI, have never been investigated.

In the present study, we compared lung-protective ventilation to traditional ventilation techniques, using a well-established porcine model of hemorrhagic shock. The focus of the study was to determine whether lung-protective ventilatory methods reduced pulmonary inflammation, as measured through the accumulation of inflammatory markers within bronchoalveolar lavage (BAL) fluids. We hypothesized that lung-protective ventilation would reduce pulmonary inflammation as compared with traditional ventilation techniques.

3.0 MATERIALS AND METHODS

3.1 Animal Housing and Preparation

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati and performed in accordance with National Institutes of Health guidelines for animal care. Female Yorkshire pigs were obtained from Isler Genetics (Prospect, OH) and housed in our animal facility. All pigs were acclimated to a 12-hour light-dark cycle for at least 48 hours, and weighed 38 to 50 kg prior to experimentation. Animals were housed alone or in pairs in cages with standard bedding, and fed and watered *ad libitum*. Food was withheld on the night before the procedure in order to prevent aspiration.

Animals were sedated with intramuscular tiletamine hydrochloride (*Telazol*, 5 mg/kg) and xylazine hydrochloride (5 mg/kg) obtained from Henry Schein Animal Health (Dublin, OH). Sedated pigs were orotracheally intubated and placed in a supine position, then mechanically ventilated using Ohmeda 7000 standard anesthesia ventilator (Madison, WI). Ventilator settings were initially set at fraction of inspired oxygen of 1.0, positive end-expiratory pressure of 5 cm H_2O using a Vital Signs positive end-expiratory pressure valve (CareFusion, Yorba Linda, CA), inserted where the expiratory limb of the ventilator circuit attaches to the ventilator, and TV of 9 ml/kg. Respiratory rate was adjusted to target an end-tidal carbon dioxide of 35±5 mmHg. Fraction of inspired oxygen was maintained at 1.0 for all animals throughout the study to prevent hypoxia and maintain consistency in supplied oxygen. Post-intubation anesthesia was achieved with inhaled isoflurane (Henry Schein Animal Health, Dublin, OH) for the duration of the study.

3.2 Hemodynamic Monitoring and Laboratory Values

Animals were shaved and prepped with Betadine solution and alcohol. Open surgical cut-down cannulation was performed to expose three vessels. The right femoral artery was accessed with a 20-gauge Arrow catheter (Teleflex Inc, Research Triangle Park, NC) for hemodynamic monitoring. The right carotid artery was cannulated with a large-bore (16G) catheter for controlled hemorrhage (BD Angiocath, Benton and Dickinson Co, Franklin Lakes, NJ). The right external jugular vein was accessed with an 8 French introducer, and cannulated with a 7.5 French pulmonary artery catheter (Edwards Lifesciences, Irvine, CA) for monitoring of mean pulmonary

artery (PA) pressure, pulmonary capillary wedge pressure (PCWP), cardiac output (CO), and central venous pressure (CVP).

Following placement of all monitoring devices, animals were then randomized to ventilation with either low TV or high TV (Figure 1). Low and high TV was calculated from animal weight at 6 ml/kg and 12 ml/kg respectively. After adjustment of TV, pigs were observed for an equilibration period of 10 minutes. This post-equilibration period was considered the baseline time point. Respiratory rate was adjusted throughout the study to maintain a pH of 7.35 – 7.45. For animals randomized to pressure-controlled hemorrhage, blood was drawn at a rate of 100 ml/min until a mean arterial pressure (MAP) of 35±5 mmHg was achieved. This was considered time zero. Fresh whole blood (FWB) was collected using heparin-coated tubing and collected in sterile blood bags coated with citrate phosphate dextrose adenine anticoagulant (Kruuse Inc., Langeskov, Denmark). Collected blood was placed on a fixed-speed nutator device to prevent inadvertent clotting. Hemorrhaged animals were maintained at a MAP of 35±5 mmHg for one hour, referred to as the shock phase. After the shock phase, pigs were randomized to resuscitation with either autologous FWB transfusion or 6% hetastarch (Hextend) solution (Moore Medial, Farmington, CT). FWB was transfused through a Purecell Neo Neonatal High Efficiency Leukocyte Reduction Filter from Pall Corporation (Port Washington, NY). Resuscitation was continued until a MAP of 50±5 mmHg was achieved. Pigs were then monitored for three hours post-resuscitation, then euthanized. All animals had a continuous infusion of lactated Ringer's solution at 25 ml/hour to maintain catheter patency for the duration of the study. The total volume infused over the experimental period was measured and recorded. Standard vital signs were recorded every thirty minutes, including heart rate, MAP, rectal temperature, respiratory rate, and oxygen saturation. PA catheter measurements were also taken every thirty minutes, and included mean PA pressure, PCWP, CVP, and CO. VetScan iSTAT point-of-care analyzer (Abaxis, Union City, CA) was used to collect data on arterial blood gases, serum electrolytes, and hemoglobin fractions. iSTAT values were collected at baseline, at time zero, and every hour until euthanasia was performed. Bronchoscopy was also performed at time zero and hourly afterwards, using Ambu aScope 3 Regular, 5.0 mm bronchoscope (Columbia, MD). The right caudal lobe bronchus was accessed for each pig to maintain consistency, injected with 15 ml of sterile 0.9% saline, and collected into a sterile flask for analysis. The bronchoscope was flushed with 50 ml of sterile saline between each BAL to prevent cross-contamination of samples. BAL specimens were analyzed for white blood cell counts and populations (Coulter AcT 10 Hematology Analyzer, Beckman Coulter, Brea, California)

3.3 Experimental Groups

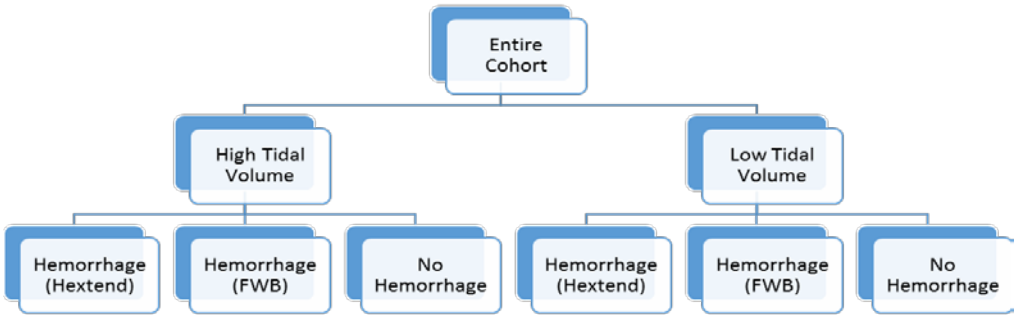

Figure 1. Tissue and serum analysis.

After the pigs were euthanized, a left thoracotomy was performed and lung samples were obtained for further analysis. Two tissue samples were obtained and immediately frozen from both the (left upper lobe) LUL and (left lower lobe) LLL for cytokine and tissue analysis. Two more tissue samples were obtained from each lobe and fixed in neutral buffered formalin for histological analysis. Lung samples were stained with a porcine anti-neutrophil antibody (T3503, BMA Biomedicals, Augst, Switzerland) to identify neutrophils. Andy's algorithm [13] was used to calculate the percent of neutrophils per slide.

Whole blood was collected and placed in serum separator tubes (BD Bioscience, San Diego, California), centrifuged at 1,000 g for 10 minutes and serum was collected. Serum levels of porcine surfactant associated protein D were measured by enzyme-linked immunosorbent assay according to the manufacturer's protocol (MyBioSource Inc., San Diego, California).

3.4 Statistical Analysis

All reported in means ± standard deviation where applicable. Statistical comparisons were performed using Kruskal-Wallis test for nonparametric variables with *post hoc* pairwise comparisons. Prism 6 (GraphPad Software, La Jolla, California) was used for all statistical analyses. Probability values of less than 0.05 were determined to be statistically significant.

4.0 RESULTS

4.1 Hemodynamics

In the low TV group, HR trended towards elevation in the groups that underwent hemorrhage, although these differences did not reach statistical significance (Figure 2). In the high TV group, the average HR in hemorrhaged pigs was significantly higher during the shock period as compared to sham, and this difference persisted until four hours post-shock (Figure 3).

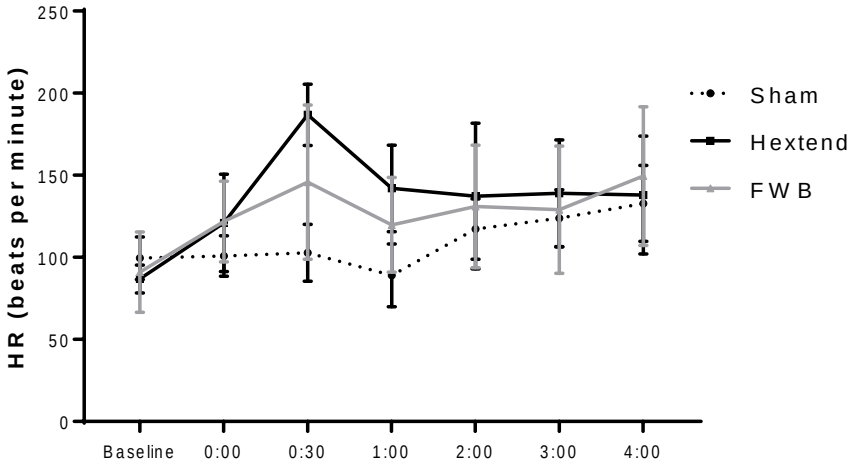

Figure 2. Low tidal volume groups' HR over time.

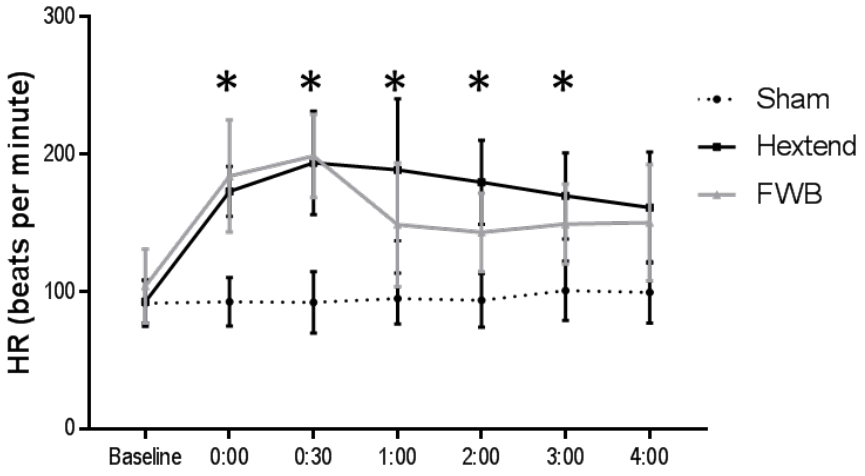

Figure 3. High tidal volume groups' HR over time.

MAP was lower in the hemorrhagic shock groups at the 0- and 30-minute time points regardless of resuscitation, consistent with the hemorrhage model design (Figures 4 and 5). CO was higher in the Hextend group compared to FWB at two hours in the low TV group (Figure 6), and compared to sham at baseline, two- and three-hours in the high TV group (Figure 7). There were no significant differences in CVP or PCWP between groups. Taken together, these results demonstrate that the physiologic effect of hemorrhagic shock in our porcine model is reflective of human physiology and consistent with class III hemorrhagic shock.

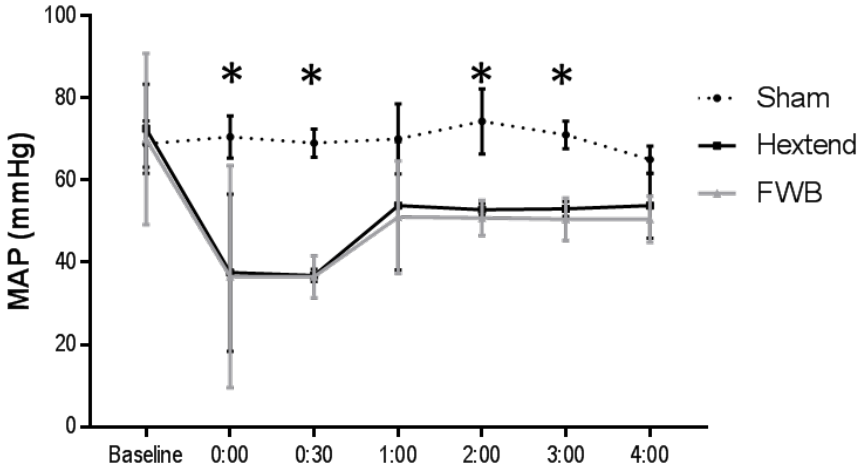

Figure 4. Low tidal volume groups' MAP over time.

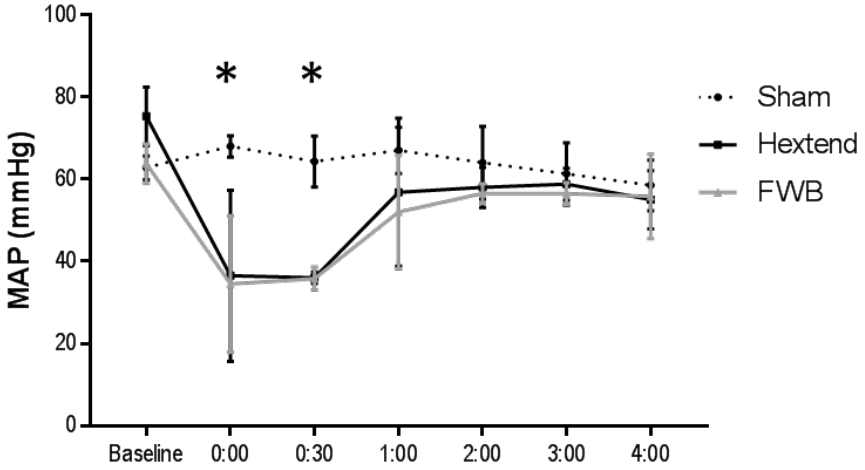

Figure 5. High tidal volume groups' MAP over time.

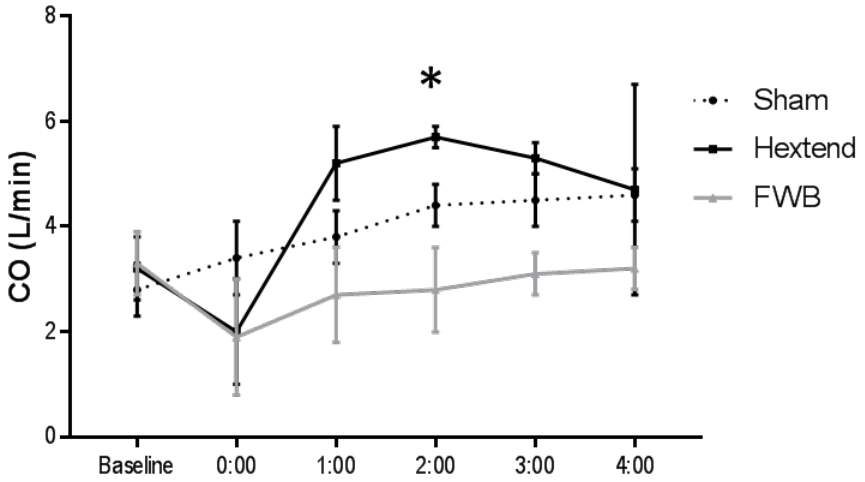

Figure 6. Low tidal volume groups' CO over time.

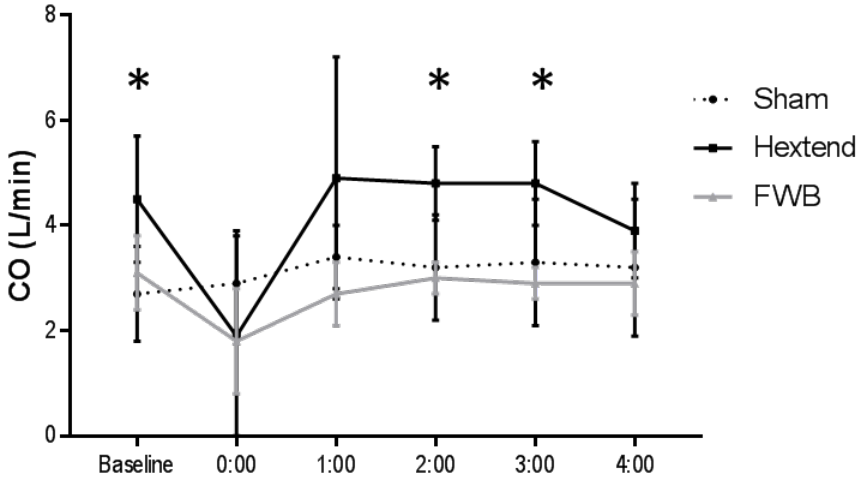

Figure 7. High tidal volume groups' CO over time.

4.2 Physiologic Response

At one hour following hemorrhagic shock, pH was lower in the Hextend-resuscitated pigs compared to sham in both low TV (7.2±0.1 vs. 7.4±0.0, p<0.01) and high TV groups (7.2±0.1 vs. 7.5±0.0, p<0.01). Similarly, FWB-resuscitated pigs were more acidotic in the high TV group compared to sham at two hours post-shock (7.4±0.1 vs. 7.5±0.1, p<0.01). Other comparisons between groups on arterial blood gas demonstrated no difference. These results confirm the reproducibility of metabolic acidosis resulting from hemorrhagic shock in this porcine model.

4.3 Resuscitation Outcomes

Complete blood counts were obtained at all time points. At low TV, hemoglobin was significantly lower in Hextend-resuscitated pigs as compared to sham at all post-shock time points (9.2±1.0 vs 5.1±0.1 g/dl, one hour; 10.2±2.1 vs. 5.8±0.1 g/dl, two hours; 10.4±1.5 vs. 5.9±1.1 g/dl, three hours; 10.3±1.4 vs. 6.5±0.1 g/dl, four hours; p<0.01 each). By comparison, hemoglobin concentration was similar between FWB-resuscitated pigs and sham pigs at all time points. These data demonstrate that Hextend resuscitation results in a dilutional anemia compared to resuscitation with FWB. The overall volume of resuscitation to maintain the target MAP was similar between Hextend and FWB-transfused groups.

4.4 Pulmonary Outcomes

In the next set of analyses, pulmonary neutrophil infiltration was evaluated using immunohistochemistry with an antibody for mature neutrophils within lung parenchyma. These results are seen in Figures 8-10. In the low-TV subgroup analysis, pigs that underwent hemorrhagic shock had a significant increase in lung neutrophil accumulation compared to sham pigs in all LLL samples (18.1±2.4%, Hextend vs. 8.8±0.9% sham; 19.6±3.2% FWB vs. 8.8±0.9% sham; p<0.001 each). These results were consistent in LUL samples as well, in both FWB-resuscitated (11.5±1.3% FWB vs. 7.5± 0.7% sham; p<0.001) and Hextend-resuscitated

pigs (15.2±2.2% Hextend vs. 7.5±0.7%, sham; p<0.001). Pulmonary neutrophil infiltration was also seen in high-TV subgroup analysis. The percent of neutrophil infiltration was significantly increased in LLL of pigs that underwent shock and were resuscitated with FWB (11.9±0.9% FWB vs. 7.6±1.4% sham; p=0.01). Similar trends were found in the LUL, however these data did not reach statistical significance (12.9±2.7% FWB vs. 7.0±1.5 % sham; p=0.07). Taken together, the neutrophil infiltration data demonstrate that hemorrhagic shock and resuscitation increases pulmonary inflammation similarly in both Hextend and FWB resuscitation groups. In addition, a low TV ventilation strategy appears to exacerbate acute pulmonary inflammation compared to high TV, and this effect is more apparent in animals resuscitated with Hextend.

BAL specimens did not show any difference in white blood cells, neutrophils, lymphocytes or monocytes. Moreover, there were no differences in levels of serum surfactant protein D between groups at any time points, and these levels did not change over time in each respective group.

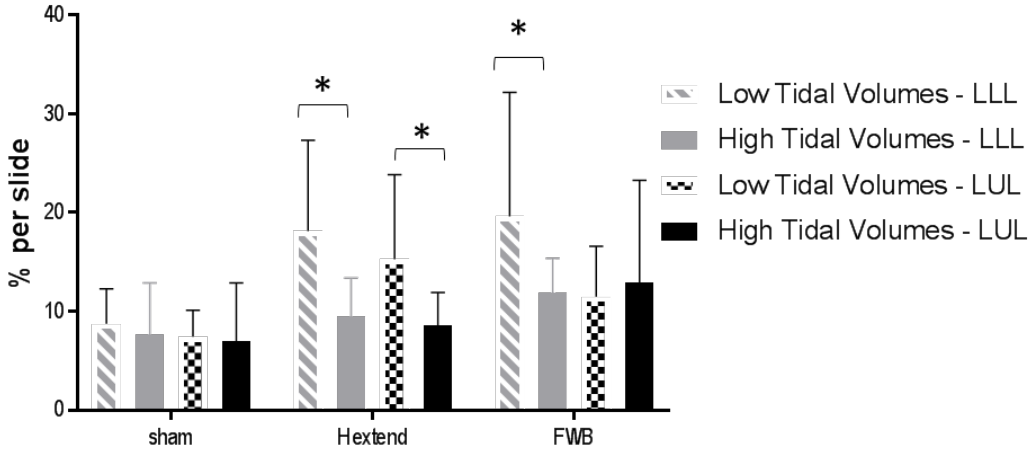

Figure 8. Neutrophil staining percentage per slide.

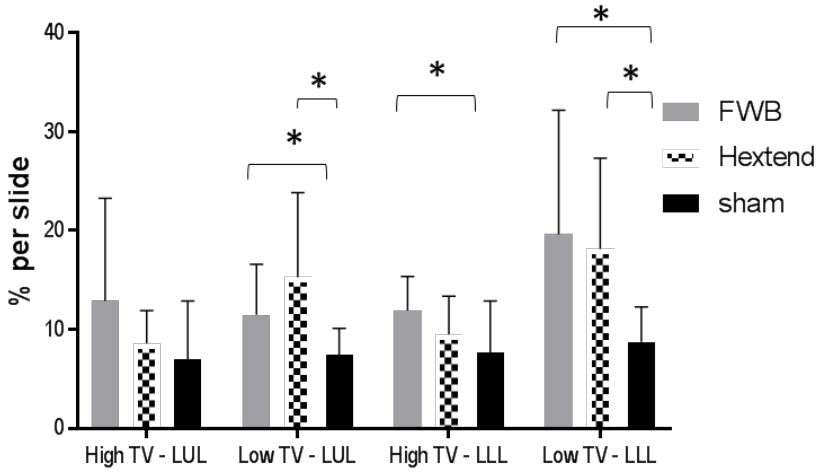

Figure 9. Neutrophil staining percentage per slide.

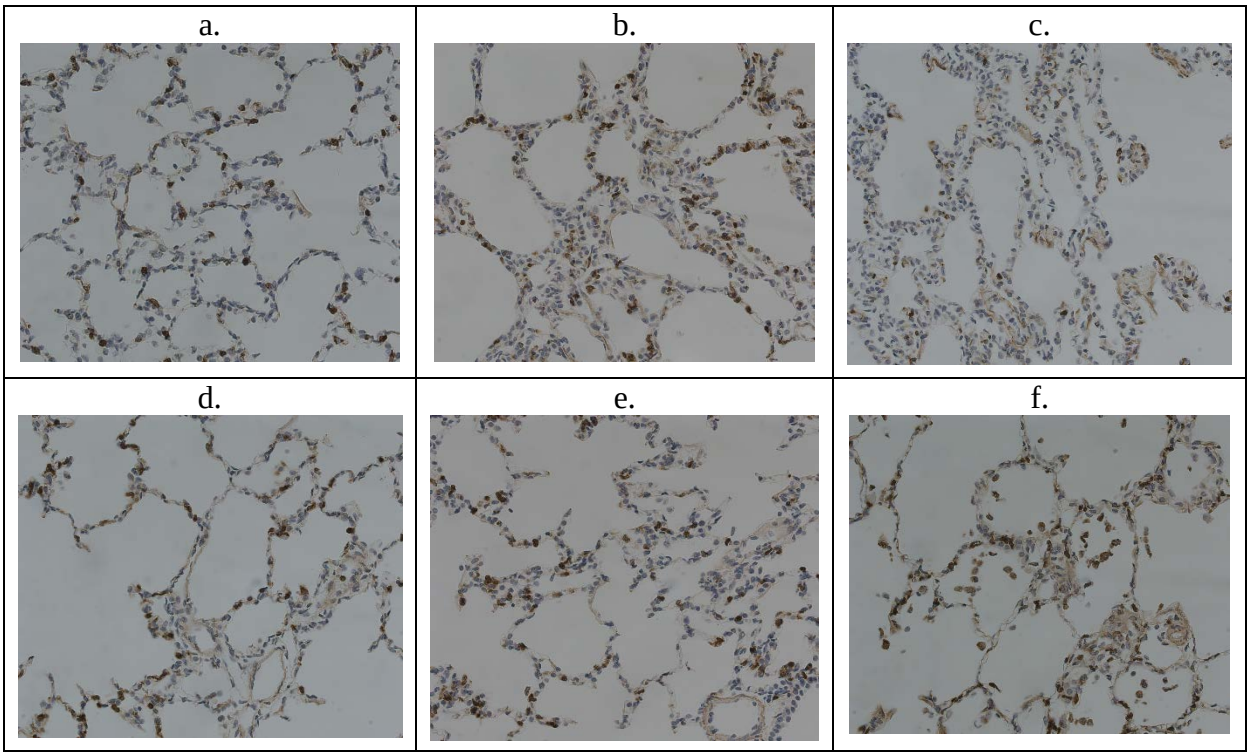

Figure 10. Neutrophil staining: a) Low TV sham b) Low TV Hextend c) Low TV FWB d) High TV sham e) High TV Hextend f) High TV FWB.

5.0 DISCUSSION

ALI and ARDS are common complications among trauma patients that lead to significant morbidity and mortality [1, 14, 15]. Lung-protective ventilation strategies can be utilized to prevent the development of ALI among these patients [16, 17]. Moreover, lung-protective ventilation using low TV has been shown to decrease overall mortality among patients who develop ARDS [12, 18]. While these strategies may mitigate pulmonary insults, there is limited data on predicting which patients will go on to develop ALI or ARDS, and who would benefit from the implementation of preventative measures early in the post-injury course. In the current study, we discovered a significant increase in pulmonary inflammatory markers among swine undergoing hemorrhage. However, we were unable to reveal any pulmonary differences with regard to early post-resuscitation ventilation strategy.

Unintentional injury is the leading cause of death in patients under 44 years old in the United States [19]. Among the traumatically injured, hemorrhage accounts for approximately 40% of preventable deaths in the civilian population and up to 90% in the military population [20, 21]. The use of damage control resuscitation strategies with limited crystalloid resuscitation in favor of blood products has been shown to improve survival rates in both of these populations [22-24]. In other studies, plasma and crystalloid resuscitation have been found to be independent risk factors for developing ARDS [25]. Massive transfusion has also been associated with pulmonary dysfunction and increased risk of post-traumatic death [26]. Despite these severe and morbid consequences, clinical or serum biomarkers have yet to be elucidated for identifying trauma patients at risk for the development of ALI.

Our group has previously utilized a successful porcine hemorrhage and lung injury model [27]. In the current study, we demonstrated the reproducibility of inducing moderate-to-severe hemorrhagic shock in a porcine model. This model induced a similar physiologic response to hypovolemic shock as seen in humans. We demonstrated successful induction of a shock state with a significantly lower MAP in the hemorrhagic shock groups compared to sham. Porcine HR after shock was elevated compared to sham models, allowing for cardiac output compensation. Hemoglobin concentrations were lower among Hextend-resuscitated pigs, reflecting an expected dilutional effect compared to whole blood resuscitation. These findings confirm the overall validity of our porcine hemorrhagic shock model and show a response similar to what is seen in patients following traumatic hemorrhage.

After confirming our porcine model, we next looked at various pulmonary inflammatory markers as potential predictors of ALI after hemorrhage. In previous studies, our group has found an increase in neutrophils in lung tissue following hemorrhage through a murine model [28]. Therefore, our initial set of experiments sought to determine whether this finding could be reproduced in pigs. Neutrophils were identified from lung samples using immunohistochemistry to compare pulmonary inflammation between groups. We found that pigs subjected to hemorrhage had increased pulmonary neutrophil infiltration compared to the control group, reflecting a shift towards a pro-inflammatory state within the lung parenchyma. Surprisingly, this effect was independent of lung region and the method of resuscitation. In addition, pulmonary inflammation appeared to be exacerbated by low TV ventilation, with a more apparent effect in the Hextend resuscitated animals. More profound effects on acute post-traumatic lung inflammation may have been found if using crystalloid rather than colloid or blood-based resuscitation techniques, as crystalloid has been shown to be a significant contributing factor post-hemorrhagic ALI incidence.

The finding of early post-injury pulmonary inflammation is likely related to the pathophysiology of trauma-related ALI, as the hemorrhagic shock state causes neutrophil diapedesis within the pulmonary vasculature. To our knowledge, this is the first paper using immunohistochemistry to identify neutrophils in a porcine hemorrhage/lung injury model. Balkamou et al. previously demonstrated decreased lung edema and microvascular permeability in swine resuscitated with Hextend compared to Lactated Ringer solution. In comparison with our findings, they also showed significantly increased pulmonary neutrophil sequestration in the Lactated Ringers group when compared to colloid resuscitation [29]. In a separate study, Roch *et al*. showed increased neutrophil infiltration and BAL inflammatory markers in hemorrhagic shock without a difference between resuscitative fluid, similar to our present investigation [30].

Next, we investigated neutrophil translocation from the pulmonary microvasculature into the alveolar sacs, through the use of BAL. The use of BAL is a common clinical practice to diagnose and treat a variety of pulmonary pathology. Previous studies have identified elevations of TNF-α and other cytokines in BAL fluid as markers of a pro-inflammatory state [30]. Our investigation revealed no differences in inflammatory markers within the BAL milieu between shocked and non-shocked pigs. It remains unclear why the increased neutrophils found histologically did not correlate with BAL samples. One hypothesis is that the neutrophils remain interstitial as opposed to alveolar in these early time points. The neutrophils may translocate into the alveoli at a later time and may have been found if the study period was extended. Another possibility is that the hemorrhage in our model was not severe or long enough to cause an inflammatory response significant enough to allow for neutrophil translocation. A more pronounced shock state would have required a larger resuscitation volume and a greater risk for

transfusion-associated lung injury [26]. These findings warrant further investigation since BAL is a relatively low risk and less invasive compared to lung biopsy to obtain histology.
Our findings should be taken with regard to several key limitations. First, we obtained samples and performed the experiment over a four-hour time-period following the initiation of hemorrhagic shock. This interval may be too short and differences may have become apparent if the study was extended for a longer duration. Second, the porcine inflammatory response may not be identical to the response observed in humans. While most studies used similar tidal volumes, the optimization and effects of low and high tidal volumes in a porcine model are unknown. Third, a more severe hemorrhage requiring a larger resuscitation may have demonstrated the significant inflammatory response that we expected to see. In our preliminary experiments, however, we found that prolonging a shock state or maintaining a lower shocked MAP led to much higher mortality rates. Lastly, the addition of a crystalloid-based resuscitation group may have demonstrated more profound pulmonary inflammation. However, the resuscitation groups were based on current military and civilian guidelines that recommend the minimization of crystalloid use. Crystalloid use, therefore, would have decreased clinical relevance and may have confounded the intended investigation of the effects of tidal volume strategies.

In conclusion, we demonstrate the reproducibility of our porcine model of hemorrhagic shock that is consistent with the physiologic changes in humans in moderate-to-severe hemorrhagic shock. Pulmonary neutrophil infiltration was increased in the hemorrhage groups compared to sham, with a possible exacerbation by low rather than high tidal volume ventilation strategies. Future studies of the acute pulmonary inflammatory effects of trauma should consider longer term observation periods in porcine models with the combined serum, bronchoscopic, and histologic assessments.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
CO	cardiac output
CVP	central venous pressure
FWB	fresh whole blood
HR	heart rate
LLL	left lower lobe
LUL	left upper lobe
MAP	mean arterial pressure
PA	pulmonary artery
PCWP	pulmonary capillary wedge pressure
TV	tidal volume