SCIENTIFIC ADVANCES

The Pattern of Early Lung Parenchymal and Air Space Injury Following Acute Blood Loss

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Abstract. Acute lung injury is a frequent clinical occurrence following blood loss and trauma. The nature of this injury remains poorly understood. **Objective:** To examine the relative parenchymal and intra-alveolar distribution of inflammation in a rat model of hemorrhage and resuscitation. **Methods:** Rats were anesthetized and subjected to hemorrhage followed by resuscitation with shed blood and saline. Myeloperoxidase activity of lung homogenates and cytology of bronchoalveolar lavage fluid were used to measure total lung and intra-alveolar neutrophil invasion. Extravasation of IV-administered [^{125}I]-albumin was used to determine total lung and alveolar permeability. Permeability results were analyzed using their base-10 logarithmic transformations. **Results:** 86 animals were studied. Whole-lung myeloperoxidase activity was increased (control = 0.34 ± 0.16 units, injured = 0.84 ± 0.43 units, p < 0.01), while there was no difference in intra-alveolar leukocyte counts (injured = 1.85 ± 1.30 × 10^5/mL, control = 2.44 ± 1.75 × 10^5/mL, p = 0.40), suggesting that the cellular component of the injury was more severe in the intravascular and interstitial spaces. There was a strong trend toward increased permeability in the interstitial compartment, and a significant increase in permeability in the intra-alveolar compartment (whole-lung permeability: control = −0.27 ± 0.19 units, injured = 0.10 ± 0.55 units, p = 0.06; alveolar permeability: control = −2.00 ± 0.47 units, injured = −1.32 ± 0.49 units, p < 0.01), suggesting that the loss of integrity to macromolecules was not limited to the interstitium. **Conclusion:** Hemorrhage and resuscitation resulted in an acute lung injury characterized by extravasation of intravascular protein into both the interstitium and the intra-alveolar space. Neutrophil invasion of the lung was demonstrable only in the interstitial compartment. **Key words:** shock; lung; trauma-induced ARDS; posttraumatic respiratory failure. ACADEMIC EMERGENCY MEDICINE 1998; 5:659–665

ACUTE respiratory distress syndrome (ARDS) affects at least 150,000 people in the United States each year.[1] Trauma and other illnesses associated with massive blood loss are important precipitants of ARDS, second only to sepsis in their strength of association with this severe pulmonary complication.[2] Indeed, in patients sustaining hemorrhagic shock in conjunction with long bone fractures, blunt thoracic injury, or pulmonary aspiration of gastric contents, the risk for progressive respiratory failure may reach 75%.[3]

While the clinical scenario of refractory hypoxia, bilateral infiltrates on chest radiography, and reduced pulmonary compliance is uncommon in the first hours following injury, available experimental evidence suggests that the secondary insult responsible for the development of these abnormalities begins within hours, if not minutes, of the acute event.[4,5] However, what is striking in these experimental models is that abnormalities in pulmonary compliance and oxygenation are difficult to demonstrate.[6] Another common finding in animal models, which distinguishes them from clinical experience, is a moderate-to-severe injury of interstitial structures associated with a relative sparing of alveoli. The specific distribution of injury in these 2 compartments has not previously been systematically examined.

Using a rat model of hemorrhagic shock and resuscitation, we sought to further examine the nature and distribution of acute lung injury following

hemorrhage and resuscitation. In an effort to localize the pulmonary inflammatory response, we devised a set of experiments that would examine whole-lung inflammation (a reflection of the total injury delivered to the intravascular, interstitial, and alveolar compartments) and isolated bronchoalveolar lavage (BAL) inflammatory markers (a reflection of only intra-alveolar injury). We hypothesized that acute blood loss and resuscitation would result in 1) increased pulmonary neutrophil content in both the lung parenchyma and the alveoli, 2) capillary leak as demonstrated by increased interstitial and alveolar content of normally intravascular protein, and 3) impaired lung function as measured by arterial O_2 tension and peak inspiratory pressure (PIP) (as a surrogate for dynamic compliance).

METHODS

Study Design. This was a prospective animal laboratory investigation of acute hemorrhage followed by resuscitation.

Animal Subjects. Male specific-pathogen-free Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing between 300 and 400 grams were cared for and studied in a manner that conformed to federal and institutional animal use and care standards. This protocol was approved by our institution's animal use and care committee.

Experimental Protocol

Anesthesia and Instrumentation. Subcutaneous ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg) were used for anesthesia. Animals were secured in a supine position, tracheostomized, and mechanically ventilated using a rodent ventilator (Model 683, Harvard Apparatus, South Natick, MA) set to deliver an FiO_2 of 1.0 and a tidal volume of 10 mL/kg at a rate of 70 breaths/min. Positive end-expiratory pressure (PEEP) was maintained at 2 cm H_2O. PIP and PEEP were measured continuously at the airway (Model 400 airway pressure monitor, Sechrist, Anaheim, CA).

Both carotid arteries (one for pressure monitoring and blood gas sampling and one for hemorrhage) and the left jugular vein were cannulated with 0.58-mm ID tubing (PE-50, Clay Adams, Sparks, MD). Mean arterial pressure (MAP) was measured continuously with a clinical monitor (Model 78901A, Hewlett-Packard, Andover, MD). Using a heating lamp, animal core temperature was maintained between 35 and 37°C as measured by a rectal thermometer.

Hemorrhage. A programmable syringe pump (Model 55-4143, Harvard Apparatus) withdrew 1.5

mL/kg/min of blood into a citrate–dextrose–phosphate anticoagulated syringe until an MAP of 25 mm Hg was reached. Additional blood was removed as needed to maintain hypotension for 45 minutes. The total shed blood volume was recorded.

Resuscitation. Injured animals were resuscitated with blood and saline infusions through the jugular vein. All shed blood was infused over 20 minutes. An additional 30 mL/kg of normal saline was given over the next 20 minutes. Following resuscitation, each animal was observed for 50 minutes; the entire protocol was therefore 135 minutes long.

Euthanasia. At the conclusion of the preparation, a small laparotomy was performed and each animal was briskly exsanguinated through the abdominal aorta.

Control Animals. Control animals were anesthetized, mechanically ventilated, instrumented, and monitored in a fashion identical to the handling of the experimental animals. The control animals did not undergo hemorrhage or resuscitation.

Measurements. Five sets of experiments were performed to examine capillary permeability and neutrophil invasion. Hemodynamic, PIP, and blood gas measurements were pooled from all 5 studies.

Hemodynamic, Inspiratory Pressure, and Blood Gas Measurement. MAP and PIP were recorded every 5 minutes. Arterial pH and O_2 and CO_2 tensions were measured at baseline and post-hemorrhage (45 min), post-resuscitation (85 min), and post-observation (135 min, Gem Premier, Mallinkrodt Sensor Systems, St. Louis, MO).

Specimen Collection. In experiments examining whole-lung injury markers, the thorax was opened immediately after euthanasia, and the superior and inferior venae cavae were ligated. After disruption of the left atrial appendage, 10 mL of saline, gently infused into the right ventricle, was used to flush the pulmonary intravascular volume. Following careful dissection from mediastinal structures and hilar lymph tissue, the left and right lungs were washed with an additional 5 mL of saline.

A similar technique was used to prepare the lungs in animals to be used for histology, except that 10% buffered formalin, rather than saline, was used to perfuse the lungs. These specimens were then embedded, mounted, and stained with hematoxylin and eosin.

In experiments examining bronchoalveolar injury markers, a thoracotomy was performed after euthanasia, and 8 mL of phosphate-buffered saline was instilled into the trachea. This fluid was with-

drawn and instilled twice more. The volume of lavage solution used was roughly equal to the total lung capacity of the rat, such that during lavage the airspaces throughout the lung were visibly distended with lavage fluid. This technique was therefore unlike clinical BAL, which is subject to significant sampling inconsistency. Preliminary experiments in our laboratory using total BAL protein content (as measured by the bicinchoninic acid technique, Pierce, Rockville, IL) in 10 healthy rats demonstrated very consistent protein yield (0.2235 ± 0.021 mg/mL), reinforcing our confidence that our technique allowed reproducible measurement of intra-alveolar contents.

Whole-lung Neutrophil Content (n = 10 Injured, 10 Control). Pulmonary neutrophils were measured using an assay for whole-lung myeloperoxidase (MPO) activity. MPO is an enzyme present in neutrophil cytoplasmic granules, and its presence in tissue homogenates is a sensitive and specific marker for total neutrophil content.[7] To determine whole-lung MPO activity, both lungs were homogenized and then sonicated in 100 mmol KH_2PO_4 buffer containing hexadecyltetraammonium bromide and ethylenediamine tetraacetic acid (EDTA). Following centrifugation at 2,300 g for 30 minutes at 4°C, lung supernatants were combined with o-dianisidine dihydrochloride in a hydrogen peroxide-containing buffer and placed in a spectrophotometer (DU486, Beckman Instruments, Irvine, CA). The change in sample absorbance at 460 nm over 1 minute was measured and reported as the MPO value.

Bronchoalveolar Neutrophil Content (n = 10 Injured, 10 Control). In another set of experiments, the control and hemorrhaged animals underwent BAL for quantification of intra-alveolar inflammatory cells. A hemacytometer (American Optical, Buffalo, NY) was used to count the total inflammatory cells present in the BAL. Cells were washed by centrifugation at 1,500 rpm for 5 minutes with resuspension of the cellular pellet in Hank's balanced salt solution (Gibco/BRL, Gaithersburg, PA). After 2 wash cycles, the cells were affixed to glass slides (Cytospin 3, Shandon, Pittsburgh, PA) and stained with hematoxylin and eosin. Differential counts of mononuclear cells and neutrophils were made by averaging the proportion of such cells seen over 10 high-power (400×) fields, and in every case represented at least 300 cells.

Whole-lung Permeability (n = 10 Injured, 10 Control). In a third set of animals, [125I]-labeled bovine serum albumin ([125I]-BSA) was used to study pulmonary capillary integrity. Thirty minutes prior to the end of each experiment, animals received between 750,000 and 1,000,000 counts of [125I]-BSA diluted in 0.3 mL of normal saline through the jugular vein. At the end of the experiment, an arterial reference sample was collected and euthanasia and lung harvest were performed as in the myeloperoxidase studies. The activities in both lungs and in the arterial reference, as well as background radioactivity, were measured using a gamma counter (Gamma 5500, Beckman Instruments). Preliminary experience demonstrated that results from these measurements were not normally distributed. Therefore, a base-10 logarithmically transformed whole-lung permeability index was calculated as follows:

$$\log_{10} \frac{\text{counts in lungs} - \text{counts in background}}{\text{counts in 1 mL blood} - \text{counts in background}}$$
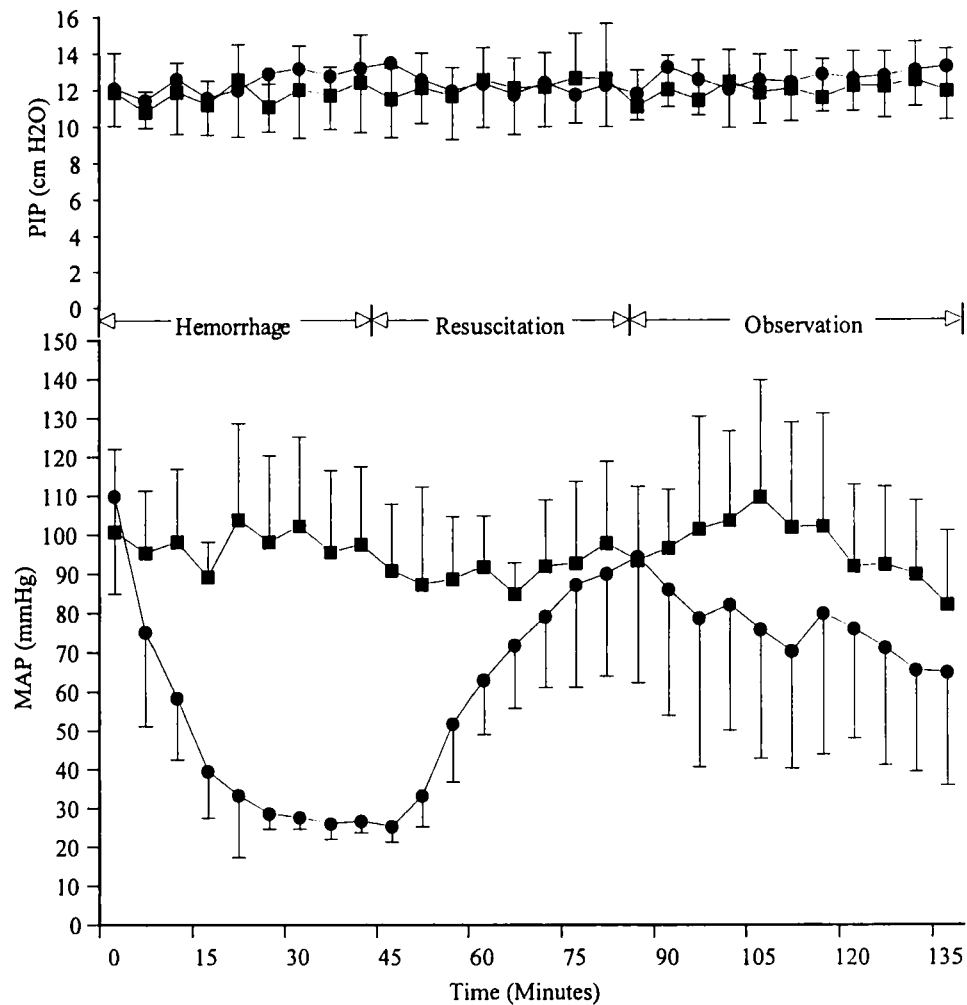
Alveolar Permeability (n = 10 Injured, 10 Control). In the fourth set of experiments, egress of radiolabeled protein into the alveolar space was measured by injecting animals with IV [125I]-BSA as in the above experiments. At the conclusion of the protocol, rather than retrieving the lungs, BAL was performed. A bronchoalveolar permeability index was calculated in a fashion similar to that for the interstitial injury index, but total counts in the BAL fluid rather than total lung counts were used in the numerator.

Morphologic Appearance (n = 3 Injured, 3 Control). In the final set of experiments, the lungs of injured animals were preserved for histologic examination. These results were intended as illustrative only, and did not receive formal numerical analysis.

Data Analysis. Descriptive statistics of MAP, PIP, and arterial blood gas data were pooled from all of the experiments, and were reported as mean ± standard deviation. Differences in gas exchange and PIP between the control and injured animals were determined with 2-sided t-tests, considering the final recorded time point only. Similarly, 2-sided t-tests were used to compare myeloperoxidase, BAL cellular content, and whole-lung and bronchoalveolar permeability index results. Sample size was determined from similar work by others in our laboratory, and p-values <0.05 were considered significant. No animal was used in >1 experiment. All statistical procedures were performed using SAS 6.11 (SAS Institute, Cary, NC).

RESULTS

Blood Loss and Hemodynamic Response. The mean blood loss among injured animals was 28.9 ± 3.7 mL/kg. Figure 1 demonstrates the hemodynamic responses of both groups. While individual

Figure 1. Hemodynamic and pulmonary mechanical performance during hemorrhage and resuscitation. PIP = peak inspiratory pressure; MAP = mean arterial pressure; squares = control group; circles = injured group. Values are mean ± SD.

responses varied overall, the injured group developed recurrent hypotension following the cessation of fluid resuscitation.

Gas Exchange and PIP. Gas exchange remained normal in both groups throughout the experiments. The mean arterial pO_2 never dropped below 425 mm Hg in either the control or injured animals. Furthermore, there were no significant differences between the groups at the end of the experimental protocol (injured = 447 ± 163 mm Hg, control = 448 ± 134 mm Hg, p = 0.98, 95%

$CI_{Injured-Control}$ = 139, −141). Injured animals were more acidotic at the conclusion of the protocol (injured = 7.28 ± 13, control = 7.37 ± 0.05, p < 0.01). A compensatory, but incomplete, respiratory alkalosis was observed (pCO_2 in injured = 30 ± 7 mm Hg, control = 35 ± 5 mm Hg, p < 0.01). PIP also did not change over the course of the protocol (Fig. 1). No difference in PIP at the final time point was demonstrable (injured = 13.5 ± 2.9 cm H_2O, control = 11.9 ± 2.3 cm H_2O, p = 0.23, 95% $CI_{Injured-Control}$ = 4.1, −0.8), suggesting that no measurable change in dynamic compliance had occurred in the injured animals.

TABLE 1. Bronchoalveolar Cytology Results

Cell Population	Control (n = 10)	Injured (n = 10)	p-value	95% Confidence Interval (Injured−Control)
Total (×10⁵/mL)	1.85 ± 1.30	2.44 ± 1.75	0.40	1.99 − −0.80
Polymorphonuclear (%)	4.39 ± 5.64	5.93 ± 5.29	0.58	7.33 − −4.25

Hemacytometry and differential counting of bronchoalveolar lavage specimens showed no difference in total cell count or the frequency of polymorphonuclear cells between control animals and animals subjected to hemorrhagic shock (reported as mean ± SD).
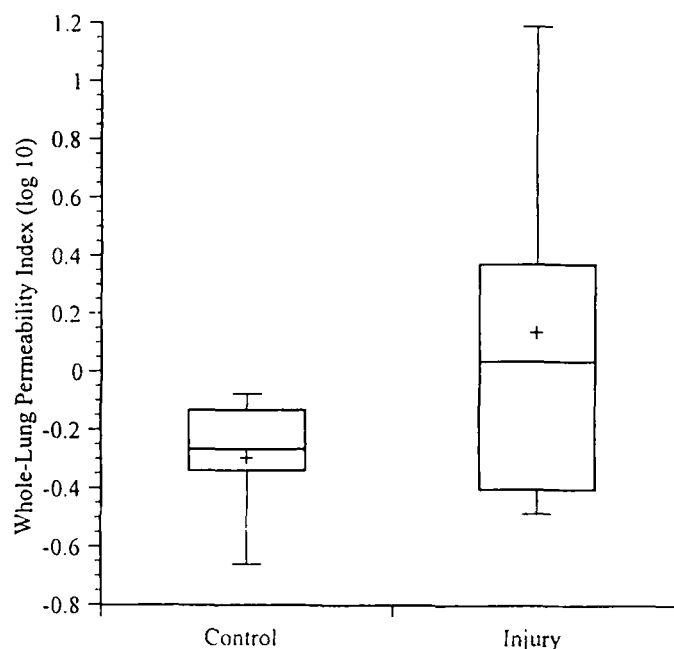
Whole-lung Neutrophil Content. Animals exposed to hemorrhage and resuscitation had a significant increase in mean whole-lung MPO activity (injured = 0.84 ± 0.43 units, control = 0.35 ± 0.16 units, p < 0.05, Fig. 2).

Bronchoalveolar Neutrophil Content. No difference in total bronchoalveolar inflammatory cell content was seen (Table 1). Cytologic examinations revealed essentially normal intra-alveolar cellular constituents, with a predominance of mononuclear cells (chiefly alveolar macrophages) and very few neutrophils in either the control or the injured animals.

Whole-lung Permeability. Acute blood loss was associated with a trend toward increased whole-lung permeability to [^{125}I]-BSA (Fig. 3, injured = 0.10, control = −0.28, p = 0.06, 95% CI$_{Injured-Control}$ = 0.86, −0.10).
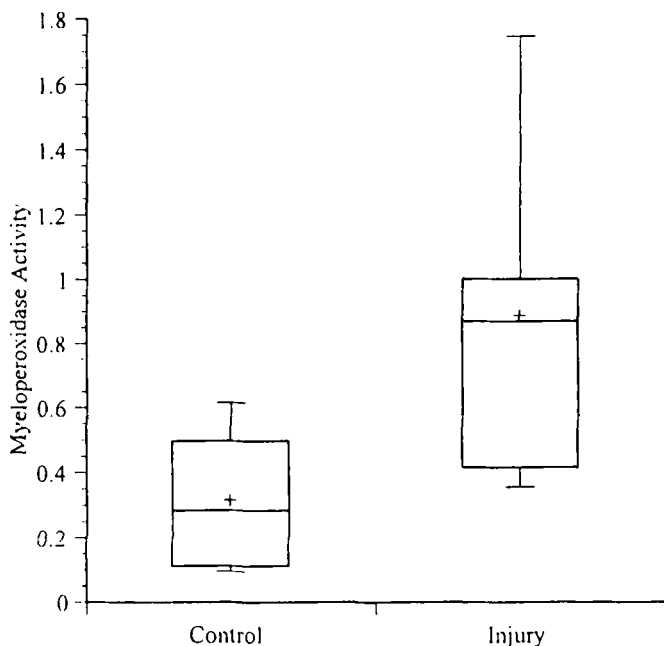
Bronchoalveolar Permeability. A significant difference was seen in alveolar permeability to radiolabeled albumin in the injured group (injured = −1.32, control = −2.00, p < 0.01, Fig. 4).

Morphologic Appearance (Fig. 5). Hemorrhagic shock and resuscitation were associated with accumulation of neutrophils in pulmonary capillaries, extensive interstitial neutrophil deposition,

Figure 3. Increase in interstitial [^{125}I]-labeled albumin following injury. Results reported as the base-10 logarithm of the ratio of whole-lung:blood radioactivity. Reported as 0th, 25th, 75th, and 100th percentiles. + = mean, p < 0.06 by 2-sided unpaired t-test, n = 10 in each group.

and intra-alveolar fibrinous exudate. Intra-alveolar neutrophils were seen infrequently.

DISCUSSION

In these experiments, we found that acute lung injury following hemorrhage and resuscitation was characterized by increased inflammatory cell content and the accumulation of intravascular protein within the pulmonary interstitium and the bronchoalveolar space. Gas exchange remained unaffected, and we were unable to demonstrate a loss of pulmonary compliance as reflected by increased PIPs.

In our model, 45 minutes of hypotension and 90 minutes of resuscitation and observation led to a >2-fold increase in whole-lung MPO activity within the lung, implying a parallel increase in the total number of neutrophils. While neutrophils are normally found in the pulmonary vascular bed, hemorrhagic shock may increase sequestration through a variety of mechanisms. The low-flow state that develops during hemorrhagic shock induces 2 rheologic conditions promoting neutrophil trapping: 1) loss of granulocyte deformability because of cellular activation in the ischemic periphery, and 2) sluggish blood flow through the lung from decreased right-sided cardiac output.[8,9] Prolonged contact with the luminal endothelial surface allows for intercellular adhesion molecules to
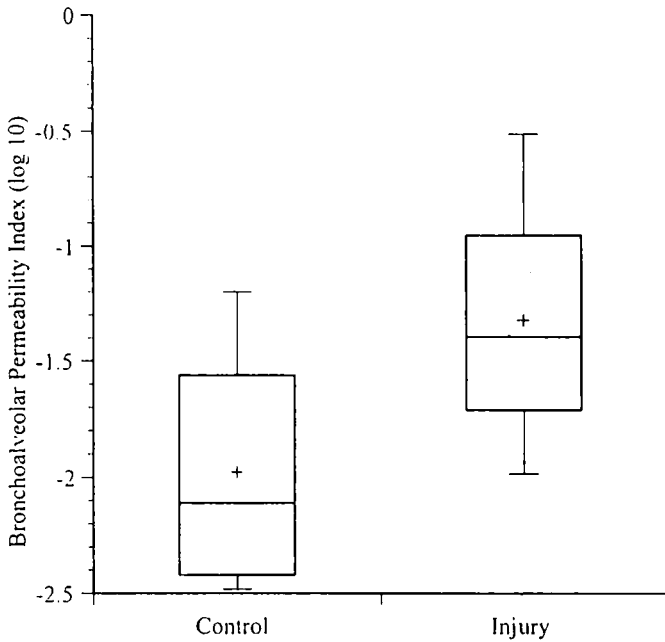
Figure 2. Pulmonary neutrophil content following injury as reflected by whole-lung myeloperoxidase activity. Reported as 0th, 25th, 75th, and 100th percentiles. + = mean, p < 0.05 by 2-sided unpaired t-test, n = 10 in each group. (Modified with permission from: Younger JG, Taqi AS, Till GO, Hirschl RB. Partial liquid ventilation protects lung during resuscitation from shock. J Appl Physiol. 1997; 83:1666–70.)
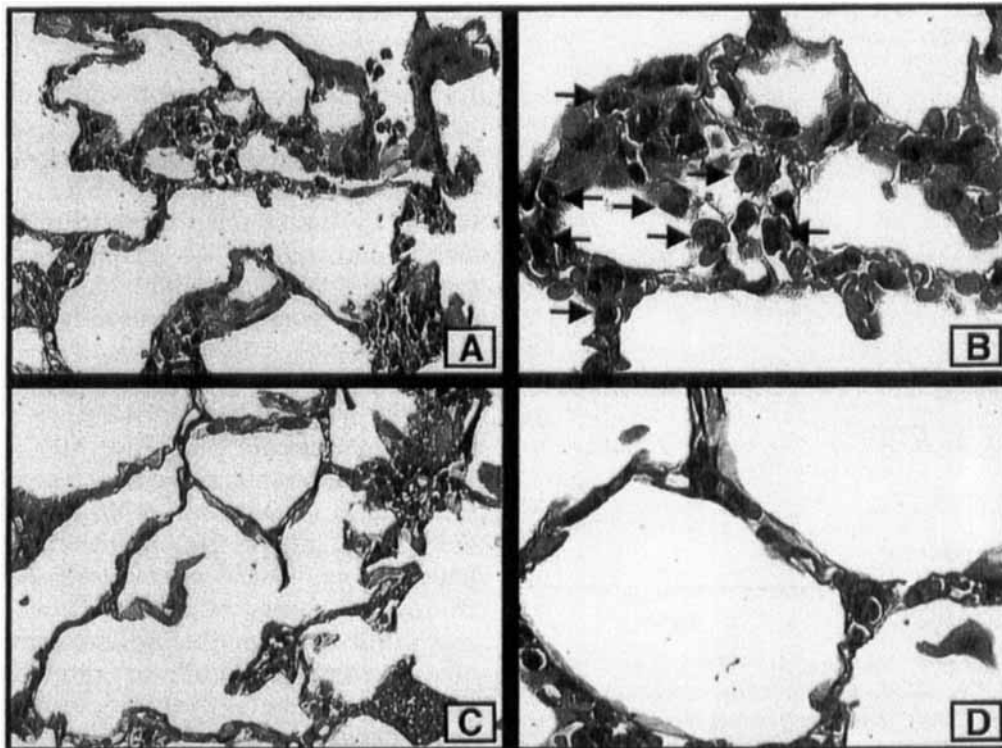
Figure 4. Increase in specific alveolar permeability as measured by bronchoalveolar lavage (BAL). Results reported as the base-10 logarithm of BAL:blood radioactivity. Reported as 0th, 25th, 75th, and 100th percentiles. + = mean, $p < 0.05$ by 2-sided unpaired t-test, $n = 10$ in each group. (Modified with permission from: Younger JG, Taqi AS, Till GO, Hirschl RB. Partial liquid ventilation protects lung during resuscitation from shock. J Appl Physiol. 1997; 83:1666–70.)

more firmly anchor neutrophils within the pulmonary intravascular space, which is the first step in tissue invasion.[10]

An important part of clinical ARDS related to trauma is the appearance of neutrophils within the alveoli of injured lung. In a series of patients suffering post-traumatic respiratory failure, Modig and Hallgren found that neutrophils comprised 55% of cells recovered by BAL, and that increasing numbers of neutrophils in BAL fluid correlated closely with the PaO_2/FiO_2 ratio, a marker of impaired oxygenation.[11] The injury produced in our model was not associated with detectable intra-alveolar invasion by neutrophils, distinguishing it both from clinical experience and from animal models of direct injury (e.g., immune complex or complement activation models, where neutrophils are often 10 times as prevalent as other cell lines in BAL fluid).

The nature and the timing of stimuli inducing egress of neutrophils out of pulmonary interstitium and into alveoli have yet to be fully delineated in hemorrhagic shock. While a longer observation period may have allowed extension of cellular migration into air spaces, recent work by Kushimoto and colleagues in a very similar rat model suggests that whole-lung MPO activity peaks between 4 and 6 hours post-resuscitation, and returns to normal by 24 hours post-event.[12]

Figure 5. Hematoxylin and eosin stains of lungs from injured and control animals. **A.** 40× view of an area of intravascular neutrophil margination and interstitial inflammation. **B.** 100× view of the same animal. Arrows indicate invading polymorphonuclear cells. **C.** 40× view of a control animal, showing preservation of the normal pulmonary interstitium and the alveolar septa. **D.** 100× view of the same animal.

The failure of neutrophils to enter the alveoli in our experiments may thus be a species-related, rather than time-related, limitation of the model.

In 1980, Demling studied the effects of hemorrhage on sheep lung. He used sheep with a chronic lymph fistula of the efferent duct of the caudal mediastinal lymph node, through which as much as 75% of ovine lung lymph passes. Demling demonstrated that the increased lung water observed after resuscitation from hemorrhage was a result of increased filtration, and not a result of increased endothelial permeability to macromolecules.[13] However, a number of other investigators have shown increased capillary permeability following hemorrhage and resuscitation.[6,14,15] Gorin and Mendiondo, using a model very similar to Demling's, demonstrated a doubling of both total lung lymphatic flow and pulmonary vascular clearance of albumin.[16] Unlike us, these authors were unable to demonstrate a change in alveolar radiolabeled albumin content following hemorrhage. To our knowledge, our current results are the first to conclusively show an increase in alveolar radiolabeled proteins following hemorrhage and resuscitation.

LIMITATIONS AND FUTURE QUESTIONS

The acute respiratory distress syndrome is a clinical phenomenon occurring over hours to days following a severe systemic injury, from infectious, ischemic, or traumatic causes. Its hallmarks of hypoxia and decreased pulmonary compliance are difficult to model in animals. In a baboon model of severe multisystem injury (MAP of 40 mm Hg for 4 hours, 50 mechanical blows to each thigh, and bilateral femur fractures), Pretorius and colleagues were unable to produce significant radiologic or gas exchange abnormalities.[4] In experiments from our laboratory incorporating major abdominal surgery, massive uncontrolled hemorrhage from a disrupted aorta, and 3 days of observation, swine appeared equally resilient to respiratory failure.[17] A major limitation of our rat model mirrors these experiences: gas exchange and pulmonary mechanics appeared normal following injury and resuscitation. The degree to which this disparity will limit further investigations into the pathophysiology of acute post-traumatic lung injury remains to be determined.

CONCLUSION

In a rat model, a period of severe hemorrhagic shock followed by resuscitation resulted in a variety of pulmonary abnormalities indicating early acute lung injury. Loss of capillary integrity was marked by extravasation of intravascular protein across the pulmonary interstitium and into alveoli, while egress of circulating neutrophils was limited chiefly to the interstitium and intravascular space. These inflammatory changes occurred without associated abnormalities in gas exchange or dynamic pulmonary compliance.

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