**AMY CARPENTER**

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**AMPLIFIED NEUTROPHIL ACTIVATION BY SEQUENTIAL HEMORRHAGE, RESUSCITATION AND PULMONARY FAT EMBOLISM: AN ANIMAL STUDY**

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**Introduction:** The objective of this study was to assess the contribution of pulmonary fat embolism caused by intramedullary femoral canal pressurization to the development of acute lung injury in the presence of resuscitated hemorrhagic shock. This clinically relevant model will allow us to evaluate the potential of fat embolism in enhancing lung injury following hypotension. We hypothesize that sequential insults of hemorrhagic shock, resuscitation and pulmonary fat embolism lead to pulmonary dysfunction which is greater than either injury alone, as indicated by the following parameters: an amplified initiation of a systemic inflammatory response and a greater spectrum of diffuse lung inflammatory reaction.

**Materials and methods:** All animal procedures were approved and performed in accordance with the local animal care committee guidelines. Following anesthetic administration and preparation, NZW rabbits were randomly assigned into one of four groups: resuscitated hemorrhagic shock (HR/FE), resuscitated hemorrhagic shock (HR), fat embolism (FE), and control. In the HR/FE group (n=6), hypovolemic shock was induced via constant carotid bleeding into a polypropylene syringe (containing 3.8% sodium citrate). Mean arterial pressure (MAP) was maintained at 30-40 mmHg for one hour. Animals were resuscitated with the entire volume of shed blood titrated with an additional volume of saline necessary to restore baseline MAP values. Following a one-hour stabilization period, we exposed both distal femoral condyles through a medial parapatellar approach to the right knee in preparation for fat embolism induction. After drilling into the intramedullary cavity in a retrograde fashion, we successively reamed the canal using 3.5, 4, and 4.5 mm diameter T-handle reamers. The intramedullary canal was pressurized via a standardized injection of 1-1.5 ml of low viscosity bone cement. The patella was then reduced and the incision was closed. In the HR group (n=6), after the one-hour stabilization period following resuscitated shock, a sham knee incision was made, but was closed immediately without drilling, reaming or pressurization. In the FE group (n=8), prior to fat embolism induction, the animals were ventilated for a 3-hour period. This time corresponds to the time it took to establish shock, resuscitate and stabilize the animals in the HR group. In the Control group (n=7), following the 3-hour ventilation period, a sham knee incision was made, but was immediately closed without further manipulations. Animals were mechanically ventilated for 4 hours post-surgical closure.

For flow cytometric evaluation of neutrophil activation, 0.5 ml of blood was mixed with CTAD at a final concentration of 1:10. Samples were stained with conjugated monoclonal antibodies against CD45 (FITC), CD11b (biotin) and then a PE-conjugated streptavidin antibody. Neutrophils were identified from other white blood cells by characteristic CD45 fluorescence and light scatter properties. A minimum of 10,000 events was acquired for each sample using a FACSCalibur model flow cytometer equipped with a 488 nm argon ion laser and data was analyzed with CellQuest software.

Following the 4-hour monitoring period, the rabbits were sacrificed by an intravenous overdose of pentobarbital. A postmortem thoracotomy was performed and the left lung was fixed in inflation with 10% buffered formalin at a pressure of 25 cm of fixative. Following fixation, the sample was sectioned sagittally and three stratified random blocks of known size were taken from the mid-sagittal slice of each lung. The specimens were embedded in paraffin, processed for histological examination and cut at 5µm thickness.

We used the two way repeated measures ANOVA to analyze data from sequential continuous variable measurements. When p<0.05, differences between groups at each time point were compared using one way ANOVA and adjusted with the Bonferroni correction. Differences between sequential time points within groups were compared using a one way repeated measures ANOVA followed by Fisher’s PLSD post hoc test. For histological analysis, a Kruskal-Wallis test was used to compare differences between groups. When p<0.05, Mann-Whitney U test was used to compare differences between groups. Adjustment for multiple comparisons was performed with Bonferroni procedure.

**Results:** Three animals died in the HR/FE group immediately following canal pressurization and were excluded from the study. We assessed neutrophil activation via CD11b mean channel fluorescence (MCF) increase as compared to baseline. CD11b MCF was only significantly elevated in the HR/FE group at 2 and 4 hours post knee manipulation. Table 1 demonstrates a quantitative analysis of lung injury in all four groups 4-hours post knee manipulation. Greater infiltration of alveoli by polymorphonuclear leukocytes as compared to control group was only significantly higher in the HR/FE group.

**Discussion:** Our histological findings support our previous findings that FE by itself does not cause lung injury, as there were no apparent differences in our histological markers of lung injury between the control and FE animals. However, the HR and HR/FE groups revealed a higher number of infiltrating neutrophils into the alveolar spaces than the controls, and in the HR/FE group this increase was statistically significant. This finding is significant because the extent of neutrophil influx and the presence of neutrophils in alveolar lavage fluid have been previously correlated with the severity of the lung injury.

Molecular markers, such as CD11b expression (a cell surface adhesion molecule that is upregulated on activated neutrophils) can be used as an early indicator of acute inflammatory reactions preceding lung injury. In fact, CD11b expression on circulating neutrophils has been previously shown to increase 6 and 12 h after major trauma and on monocytes after primary hip arthroplasty. Our white blood cell flow cytometric studies complement our histological findings and demonstrate CD11b mean channel fluorescence measurements to be significantly higher only in the HR/FE group at 4 hours post knee manipulation as compared to baseline. In support of our hypothesis, the combined HR/FE did elicit two significant synergistic responses: greater neutrophil activation and enhanced alveolar infiltration. These parameters were not elevated in the FE or HR groups alone. These findings indicate the initiation of an inflammatory response when resuscitated shock is combined with fat embolism and may play a role in the development of FES.

**Table 1 - Histological assessment of lung injury 4 hours following knee manipulation (median + range; *denotes p<0.05 as compared to control)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Alveolar Hemorrhages</th>
<th>Alveolar PMNs</th>
<th>Alveolar edema</th>
<th>Hyaline membrane formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 (1-2)</td>
<td>2 (0-5)</td>
<td>1 (0-3)</td>
<td>1.5 (0-3)</td>
</tr>
<tr>
<td>Fat embolism</td>
<td>1 (0-2)</td>
<td>2 (0-5)</td>
<td>0 (0-2)</td>
<td>1 (0-3)</td>
</tr>
<tr>
<td>Shock</td>
<td>2 (1-3)</td>
<td>3 (1-7)</td>
<td>1 (0-3)</td>
<td>1 (0-3)</td>
</tr>
<tr>
<td>Shock+ FE</td>
<td>1.5 (1-3)</td>
<td>3 (1-6)</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
</tr>
</tbody>
</table>

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