High Concentrations of Reactive Oxygen Species in the BAL Fluid Are Correlated with Lung Injury in Rabbits after Hemorrhagic Shock and Resuscitation

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Increased levels of cytokines or reactive oxygen species (ROS) in the bronchoalveolar lavage (BAL) fluid are associated with acute lung injury after ischemia/reperfusion. We investigated the correlation of these markers with the degree of lung injury in a rabbit model of hemorrhagic shock. Rabbits, maintained by mechanical ventilation, were left untreated (control) or subjected to hemorrhagic shock by withdrawing blood (n = 12 for each group). Shock animals were re-infused their shed blood for resuscitation. At the end of the experiment, BAL fluid was recovered, in which parameters of oxidative stress and cytokines were measured. Macrophages and malondialdehyde levels were increased (p = 0.043 and p = 0.003, respectively), and total antioxidant capacity (TAC) was decreased in the shock animals compared with control (p = 0.009). Production of ROS was significantly enhanced in shock animals compared with controls (p < 0.001). BAL fluid levels of tumor necrosis factor-α, interleukin (IL)-1β and IL-6 were higher in shock rabbits by more than twofold (p < 0.001 for each). Shock animals also showed higher histopathological scores that represent severe tissue damage than controls (p = 0.022). Numbers of macrophages and levels of ROS and TAC were correlated with the degree of lung injury (p = 0.006, p = 0.02, and p = 0.04, respectively), but not cytokines. Therefore, resuscitation from hemorrhagic shock results in acute lung injury, with enhanced pulmonary oxidative and inflammatory responses. In conclusion, ROS in the BAL fluid are good markers that predict lung injury following hemorrhagic shock and resuscitation.

Acute respiratory distress syndrome (ARDS), which represents the most severe form of acute lung injury (ALI), is an acknowledged consequence of hemorrhagic shock, through a complex cascade of events including the effects of hemorrhage itself and the host responses (Regel et al. 1996; Rixen and Siegel 2000). The multi-factorial pathway leading to lung injury has been the subject of intensive immunologic research. Many pathophysiological aspects have been clarified, underlining the importance of certain cell populations such as polymorphonuclear leukocytes (PMNs) or even platelets (Twardy et al. 2006) in inducing intense inflammatory reactions. The initiation of the inflammatory cascade culminates in increased cytokine production (Suter et al. 1992; Meduri et al. 1995) and reactive oxygen species (ROS) formation (Baldwin et al. 1986), suggesting potential future interventions (Hasko et al. 2006).

Although oxygen free radicals have been shown to play a crucial role in the induction of lung injury, research on ROS has been marred with difficulties in their measurement. Cytokines can be readily detected in the bronchoalveolar lavage (BAL) fluid (Shenkar et al. 1994); on the other hand, ROS are difficult to be measured since they are rapidly produced and disappear, particularly those formed in biological fluids of low cell number like BAL fluid. Numerous assays provide only indirect evidence of ROS activity. Some of these indirect methods include the consumption of endogenous antioxidant substances such as glutathione, superoxide dismutase, catalase and biochemical markers of oxidative status or markers of lipid peroxidation.

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(malondialdehyde). Recently, flow cytometry has been proposed (Setsukinai et al. 2003) as a useful alternative to measure intracellular ROS, and has been applied in limited experimental studies focusing on lung pathology (Voter et al. 2001; Zeidler et al. 2003).

There has been only one study in the past focusing on the combined detection of cytokines and ROS in the BAL fluid from animals resuscitated from hemorrhagic shock (Zhang et al. 2003). The aim of our study was to provide pathophysiological insights in the implication of the oxidative and inflammatory responses in the development of ischemia-reperfusion induced acute lung injury using the well-known model of hemorrhagic shock resuscitation. For this reason we quantified markers of oxidative stress and inflammation, measuring among others ROS by flow cytometry and cytokines in the BAL fluid, and evaluated lung injury score. We further sought to identify which of these measurable markers can reliably predict the degree of lung injury.

### Materials and Methods

#### Animals

The experimental protocol was approved by the National Committee on Use and Care of Animals and the institutional Ethics Committee. Adult male New Zealand white rabbits of 3.1 to 3.4 kg body weight were used. The animals were fasted overnight with access to water ad libitum. They were anesthetized with Ketamine (35 mg/kg) and Xylazine (5-10 mg/kg) intramuscularly.

#### Experimental Set-up

A marginal ear vein was cannulated and tracheostomy was performed. Mechanical ventilation was initiated on a volume control mode using a Siemens 900 respirator, with a tidal volume of 8 ml/kg and such a respiratory rate to maintain PaCO$_2$ at 33-37 mmHg. A positive-end-expiratory pressure of 3 cm H$_2$O and a mixture of air and oxygen were administered to keep a PaO$_2$ level at 95-105 mmHg. Anesthesia was maintained using the aforementioned doses of Ketamine and Xylazine given intramuscularly every 90 min.

Left and right carotid arteries and left internal jugular vein were catheterized for blood pressure monitoring, blood withdrawal for shock induction, and reinfusion of shed blood for shock resuscitation, respectively. The central body temperature was kept between 38 and 39°C with the aid of an electrical blanket.

#### Protocol

After animal preparation was set-up, a period of 30 min was allowed in order for animals to stabilize. They were subsequently randomly allocated to two groups; animals were subjected (shock group, $n = 12$) or not subjected (control group, $n = 12$) to shock. Induction to shock was conducted by continuously collecting blood at a rate of about 1.5 to 2.5 ml per minute from the right carotid artery allowing mean arterial pressure (MAP) to gradually decrease to 40 mmHg over a total time of 30 min. Blood was poured in a reservoir containing 250 UI of heparin, gently stirred by a magnetic agitator in a 37°C bath. MAP was maintained at the aforementioned level for 60 min by withdrawing or re-infusing blood as required.

Resuscitation followed thereafter in animals of the shock group by re-infusing the whole quantity of the shed blood over a period of 30 min. From 30 to 120 min of resuscitation, Ringer lactate was administered as needed to reach, if not already achieved, and maintain MAP at 90% instead of 100% of baseline level in order to avoid lung edema. The control group animals during the experiment were simply mechanically ventilated and Ringer lactate was administered intravenously at a rate of 0.2 ml/kg/min. Animals were observed until 120 min of resuscitation since at that time-period the effect of shock resuscitation in the BAL fluid and lung histology is detectable (Gurfinkel et al. 2003; Powers et al. 2005). At the end of the experiment, animals were prepared for BAL procedure; chest was opened by midline sternotomy, exsanguination followed by cardiac puncture and euthanasia was performed synchronously with 150 mg of pentobarbital given intravenously.

#### Preparation of BAL and Measurements

The left mainstem bronchus was clamped with a hemostat. Through the right mainstem bronchus, 10 ml of saline with ethylenediaminetetraacetic acid disodium (EDTA-2Na) (final concentration 0.77 mM) at 4°C was slowly infused and the recovered fluid was discarded. Thereafter, the infusion was repeated for five additional times, the volume of harvested BAL fluid was recorded and the following measurements were performed.

A cytocentrifuged preparation (Cytospin 2; Shandon Southern Products, Runcorn, UK) of the BAL fluid was stained with Wright-Giemsa for cell differentiation. The cells present in the fluid were counted with a Coulter counter (Coulter Electronics) using the Bürker-Türk method.

A quantity of BAL fluid was centrifuged at 400 g at 20°C for 10 min, the supernatant was discarded and the pellet was re-suspended in 1ml phosphate buffer solution (PBS) for ROS measurement. This solution was preferred over saline for its pH buffering capacity. Another quantity was centrifuged at 250 g at 4°C for 10 min; the cell-free supernatant was divided into several aliquots and stored at −80°C until assayed for all other measurements.

#### (a) ROS measurement

Cells were adjusted to 1 x 10$^6$ cells/ml in PBS. ROS production was measured by flow cytometry (Víctor and De La Fuente 2003) using dichlororhydrorfluoresceine diacetate (DCFH-DA) (Molecular Probes, Oregon, USA) as a probe. It is oxidized by ROS in the cytoplasm to 2′,7′-dichlorofluoresceine (DCF), which is a highly fluorescent compound. Aliquots of 100 µl of cell suspension were incubated with DCFH-DA (0.5 mM) for 15 min at 37°C in a humidified atmosphere containing 5% CO$_2$. Aliquots undergone the procedure but not loaded with DCFH-DA, served as negative controls for determining the baseline level. Then the samples were analyzed using a flow cytometer (FACScalibur, BDBiosciences, San Jose, CA).

A two-parameter dot-plot of the side and forward scatter (SSc and FSc) height of light for the total cell population was first analyzed. Two gates were set to distinguish the macrophages and PMNs from the total cell population. For each measurement, 10,000 cells were acquired based on forward and side-scatter characteristics. ROS generation was analyzed using histogram for FL-1 (Fluorescence-1, 515-545 nm) and the results were expressed as geometric mean of the fluorescence intensity (GMFI) for the total and the gated cell populations (macrophages and PMNs). The geometric mean fluorescence channel was derived by CellQuest software (FACScalibur, BDBiosciences, SanJose, CA).
(b) Cytokine measurement

Interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) protein levels were measured by Rabbit Enzyme-Linked Immunosorbent Assay (ELISA) in the supernatant. The reagents were provided by the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). The detection range is 3-500 pg/ml for IL-1β, 3-500 pg/ml for TNF-α and 30-2,000 pg/ml for IL-6.

(c) Malondialdehyde (MDA)

The supernatant MDA measurement was performed on the Shimatzu HPLC system with fluorescence detector using the MDA Kit (Immun-Diagnostik, Bensheim). Briefly, fluid obtained after 60 min at 95°C and optimization of pH were separated with isocratic method using a reversed phase Bischoff Prontosil Eurobond column, 5 μm, and 125 × 4 mm. The chromatograms were recorded by a fluorescence detector at 515 nm excitation and at 553 nm emission, and the results were expressed as μmol/L.

(d) Total antioxidant capacity (TAC)

The supernatant TAC measurement was performed with a chemiluminescence method as previously described (Villiotou and Deliconstantinos 1995). The reaction took place in PBS 10 mM pH 8.5 on a Berthold AutoLumat LB953 luminometer. Briefly, 100 μl of fluid within 350 μl PBS were introduced into the luminometer where 100 μl of the stable free radical 2,2 azobis (2 methyl propionamidine) dihydrochloride 12.3 mM and 50 μl luminol (10 μM in Dimethyl sulfoxide) were injected. The chemiluminescence response was monitoring and the delay of the appearance of the peak was translated and the results were expressed into Trolox equivalent (mM), the soluble form of vitamin E.

Lung Histology

The left lower lobe was immersed in 10% formaldehyde solution for subsequent histopathological study. After 7 days of fixation, lungs were cut and processed for paraffin embedding. Tissue blocks of each lung were cut at 5 μm sections and stained with hematoxylin and eosin (H-E). Two pathologists, who were unaware of the animal’s treatment group, examined one slide from each case blindly and independently. The whole slide was screened and evaluated on high-power field of 0.096 mm². A total of 92 lung slides were coded and scored semi-quantitatively for the presence of inflammatory infiltration, defined as the infiltration of alveolar walls by PMNs, since it represents an early marker of acute lung injury (Gomez et al. 2007). The inflammatory score including thickness of basement membrane, congestion and edema of the alveoli, infiltration by red blood cells, inflammation of the interstitial space and inflammation of the alveoli, was evaluated using a 4-point scale. This 4-point scale was as follows: 0, none; 1, slight; 2, moderate; and 3, severe. The inter-observer variability was < 5%. In case of disagreement, the slides were re-evaluated jointly by the two observers so that a consensus could be reached.

Statistical analysis

Data are reported as median (interquartile range). Comparisons between control and shock groups were examined by the Kruskal-

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 12)</th>
<th>Shock group (n = 12)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Recovered BAL fluid (mL)</td>
<td>21 (15 - 23.7)</td>
<td>18.5 (12.5 - 23.7)</td>
<td>NS</td>
</tr>
<tr>
<td>TC/L</td>
<td>1,525 (1,308 - 1,807)</td>
<td>2,240 (1,530 - 3,279)</td>
<td>0.014</td>
</tr>
<tr>
<td>PMNs/L</td>
<td>97.8 (67.8 - 157.7)</td>
<td>88 (66.8 - 112.6)</td>
<td>NS</td>
</tr>
<tr>
<td>MCs/L</td>
<td>1,387 (1,189 - 1,608)</td>
<td>2,067 (1,714 - 3,031)</td>
<td>0.003</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>0.18 (0.08 - 0.24)</td>
<td>0.29 (0.21 - 0.30)</td>
<td>0.003</td>
</tr>
<tr>
<td>TAC [Trolox equivalent (mM)]</td>
<td>157 (116.5 - 207.7)</td>
<td>106.5 (52 - 142.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>Lung histopathologic score</td>
<td>1 (0.25 - 2)</td>
<td>2.5 (1.25 - 3)</td>
<td>0.022</td>
</tr>
</tbody>
</table>

BAL, broncho-alveolar lavage; TC, total cells; PMNs, polymorphonuclear leucocytes; MCs, macrophages; MDA, malondialdehyde; TAC, total antioxidant capacity.
Wallis test; pairwise comparisons were made by Mann-Whitney test with Bonferroni correction. Cytokine levels below detection thresholds were considered as the corresponding detection limit. The non-parametric correlation coefficient Spearman was applied to examine the relationship between histologic score and the concentrations of the parameters measured in the BAL fluid. A \( p \) value less than 0.05 was considered significant.

**Results**

Parameters of BAL fluid are presented in Table 1. It appears that in shocked animals, total cells/L and macrophages/L were significantly higher than control group, while neutrophils/L were similar between the two groups.

As mentioned in Materials and Methods, macrophages and PMNs were distinguished by the total cell population using flow cytometry (Fig. 1). Fig. 2 shows typical dot plots from BAL fluid cells of laboratory negative control, control, and shock animals (A, B and C, respectively). The fluorescence of BAL fluid cells that have generated ROS reacting and binding to the fluorescent dye is shown above the line. It is obvious that in the BAL fluid of shock animals, an abundance of ROS has been formed in contrast to the control group which resembles the negative control. Additionally, two subpopulations of cells are identified in the BAL fluid i.e. the macrophages and PMNs laid at the left and right side of each scheme, respectively. The median (range) value of GMFI quantifying ROS of shock animals was significantly enhanced compared to controls and the levels of total cells are presented in Fig. 3.

Furthermore, the median values of MDA were significantly higher in BAL fluid from shock animals, compared to controls, while a significant inverse pattern was observed for TAC values between shock and control animals (Table 1). A statistically significant increase was observed in cytokine (IL-1\( \beta \), TNF-\( \alpha \) and IL-6) levels in BAL fluid of shock animals (Fig. 4) \( (p < 0.001) \) for all cytokines.

On lung histology (Table 1), control animals exhibited
on average a slight inflammatory infiltration (Fig. 5A, median value 1, range 0-2), while shock animals exhibited on average moderate inflammatory infiltration (Fig. 5B, median value 2.5, range 1-3), a difference statistically significant ($p = 0.022$).

We further sought to investigate the existence of any statistical significant correlation between values of these measurable markers and the corresponding lung histology. Table 2 shows that the total number of macrophages, but not PMNs, and the ROS and TAC levels, but not cytokines and MDA, were statistically significantly related to the range of histological injury.

### Table 2. Relationship between histopathologic score of the lung and the concentration of various parameters in the BAL fluid applying the non parametric correlation coefficient Spearman ($r_s$).

<table>
<thead>
<tr>
<th></th>
<th>($r_s$)</th>
<th>$p$</th>
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</thead>
<tbody>
<tr>
<td>MDA</td>
<td>0.120</td>
<td>0.576</td>
</tr>
<tr>
<td>TAC</td>
<td>−0.428*</td>
<td>0.037</td>
</tr>
<tr>
<td>ROS TOTAL</td>
<td>0.468*</td>
<td>0.021</td>
</tr>
<tr>
<td>ROS PMNs</td>
<td>0.485*</td>
<td>0.016</td>
</tr>
<tr>
<td>ROS MCs</td>
<td>0.464*</td>
<td>0.022</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.227</td>
<td>0.287</td>
</tr>
<tr>
<td>TNF-α</td>
<td>−0.011</td>
<td>0.959</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.288</td>
<td>0.173</td>
</tr>
<tr>
<td>TC/L</td>
<td>0.413*</td>
<td>0.045</td>
</tr>
<tr>
<td>MCs/L</td>
<td>0.547**</td>
<td>0.006</td>
</tr>
<tr>
<td>PMNs/L</td>
<td>0.136</td>
<td>0.525</td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

MDA, malondialdehyde; TAC, total antioxidant capacity; ROS, reactive oxygen species; PMNs, polymorphonuclear leukocytes; MCs, macrophages; TC, total cells.

**Discussion**

This study shows that resuscitation from hemorrhagic shock is associated with oxidative and inflammatory pulmonary response that is quantified by ROS and cytokine increased concentrations in the BAL fluid and by lung histopathology.

With few exceptions (Zhang et al. 2003), in the case of ALI, ROS and cytokines have not been measured in combination at the site of their production, namely in the BAL fluid. Besides, the current study is the first one that investigates the biological oxidative and inflammatory pulmonary loads in relation to lung morphologic changes that follow resuscitation from hemorrhagic shock.

It is well known that hemorrhagic shock resuscitation triggers a generalized inflammatory response that is particularly prominent in the lung and contributes to posttraumatic ALI. It is characterized by accumulation of neutrophils in the lung, which represent one of the main sources contributing to ROS generation, adhesion molecule expression on the endothelium of pulmonary capillaries (Fan et al. 1998; Douzinas et al. 2009) and up-regulation of cytokines (Rixen and Siegel 2000). Studies aiming to enhance the effectiveness of shock resuscitation infusing either hypertonic saline (Powers et al. 2005) or albumin (Powers et al. 2003) have additionally shown an attenuation of ALI. Many studies support the hypothesis that ROS generation is implicated in the production of various mediators, cytokines and reactive molecules that altogether lead to ALI after shock resuscitation (Fan et al. 1998; Xu et al. 2004). Additionally, in vitro data demonstrated a direct involvement of ROS in the activation of cytokines (Matsubara et al. 1997; Jennings et al. 2004). Apart the immune cell-originated ROS however, an increment, has been also implicated for the initiation of the inflammatory cascade, coming from target tissue cells (Simon and Fernandez 2009). This increment, principally endothelial in origin,
exerts a secondary oxidative effect which acts cumulatively to the immune cell-originated ROS, representing the primary effect. In fact, endothelium may trigger endotoxin-mediated inflammatory process (Simon and Fernandez 2009). Specifically, ischemic intestine deliberates endotoxin and IL-1β in the portal blood upon reperfusion which is associated with lung injury in contrast to findings seen at the end of the preceding 120 min ischemia; similarly morphological changes are more severe after reperfusion than the end of ischemia (Douzinas et al. 2004). Inherently, we have recently shown that at the end of shock period where tissue hypoxia and hypoperfusion maximize, blood MDA remain at basic levels in contrast to the resuscitation period where they increase considerably (Douzinas et al. 2008). These data indicate that at ischemia, cells and tissues suffer and undergo priming that serves, upon O2 re-introduction, for membrane peroxidation and cell injury.

Therefore this accepted experimental model of inducing lung injury i.e. animals resuscitated from hemorrhagic shock, was chosen to quantify the oxidative and inflammatory responses of the lung. The oxidative response of the lungs in resuscitated from shock animals increased as it is signified by the higher concentration of ROS in the BAL fluid compared with controls. ROS aggressive tissue effect was further confirmed by the exhaustion of the antioxidant capacity indicated by the low TAC levels and by the synchronous increase of lipid peroxidation, an index of alveolar injury, indicated by the higher MDA concentration in the BAL fluid. Besides, a similar pattern of inflammatory response was manifested. Specifically, raised cytokine levels were observed in shock resuscitated animals that were accompanied by increased number of alveolar macrophages in the BAL fluid compared with controls. The significant difference of the inflammatory cell infiltration between groups observed in the lung histopathology indicates the parenchyma injury ensued in shock-submitted animals. Therefore, it appears that hemorrhagic shock resuscitation not only induces an immediate oxidative and inflammatory pulmonary reaction but besides, mobilizes a concurrent lung tissue inflammatory infiltration, causing ALI.

The results of this study dictate that increased detection of ROS and cytokines in the BAL fluid may announce ALI. However, the non-parametric coefficient study, as it is shown in Table 2, indicates a direct correlation of ROS and TAC with the severity of histologic score. In contrast, cytokine levels did not show such a correlation, though they significantly differed between groups. It may be suggested therefore that the extent of oxidative stress measured may predict the severity of lung injury that follows hemorrhagic shock and resuscitation.

As mentioned, direct measurement of ROS is extremely difficult because of the short lifespan of these species; this is particularly true for fluids of low cellularity like BAL fluid. Detection of ROS formation by incubating cellular suspensions with DCFH-DA has been performed in a variety of biological systems, including neutrophils from peripheral blood (Bass et al. 1983) and skeletal muscle fibers (Murrant and Reid 2001). In the current study, using the same dye as a probe, a quantitative flow cytometry assay was applied to detect ROS in BAL fluid. The method measures the total oxidative potential with the exception of O2− since this radical appears to be unable to oxidize DCFH directly (Zhu et al. 1994). In contrast, in studies attempting to assess pulmonary oxidative stress, only hydrogen peroxide was measured either in BAL fluid (Zhang et al. 2003; Xu et al. 2004) or in breath condensate (Zuckerbraun et al. 2005). Interestingly, the results from our study showed that the generation of ROS derives mainly from PMNs cells despite that macrophages are the dominant cells in the BAL fluid.

In conclusion, the results of the current study suggest that hemorrhagic shock resuscitation is associated with ALI. The combined measurement in the BAL fluid of oxidative parameters such as ROS, MDA and TAC may define pulmonary oxidative stress predicting thus overcoming ALI. Furthermore, the present study may further denote that ALI is principally associated with disruption of the oxidative equilibrium, a finding with significant pathophysiological implications in need of further confirmation.

References


