Expression of High Mobility Group Box 1 Protein in a Polytrauma Model During Ground Transport and Simulated High-Altitude Evacuation

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ABSTRACT

Background: We investigated the expression of high mobility group box 1 (HMGB1) protein in a combat-relevant polytrauma/acute respiratory distress syndrome (ARDS) model. We hypothesized that systemic HMGB1 expression is increased after injury and during aeromedical evacuation (AE) at altitude. **Methods:** Female Yorkshire swine (n =15) were anesthetized and cannulated with a 23Fr dual-lumen catheter. Venovenous extracorporeal life support (VV ECLS) was initiated via the right jugular vein and carried out with animals uninjured on day 1 and injured by bilateral pulmonary contusion on day 2. On both days, animals underwent transport and simulated AE. Systemic HMGB1 expression was measured in plasma by ELISA. Plasma-free Hb (pfHb) was measured with the use of spectrophotometric methods. **Results:** Plasma HMGB1 on day 1 was transiently higher at arrival to the AE chambers, increased significantly after injury, reaching highest values at 8,000 ft on day 2, after which levels decreased but remained elevated versus baseline at each time point. pfHb decreased on day 1 at 30,000 ft and significantly increased on day 2 at 8,000 ft and postflight. **Conclusions:** Systemic HMGB1 demonstrated sustained elevation after trauma and altitude transport and may provide a useful monitoring capability during en route care.

KEYWORDS: *acute respiratory distress syndrome; polytrauma; evacuation; altitude physiology; HMGB1*

Introduction

Ground and high-altitude evacuation of combat casualties is at the forefront of military research as it presents therapeutic and logistical challenges.[1,2] Future anti-access area denial operations will prohibit immediate evacuation of casualties because of contested airspace. This delay in evacuation, necessitating extended care-in-place for combat casualties may last from hours to days and mandate changes in practice, to include prolonged field care (PFC) of casualties.[3,4] Delivery of medical supplies to PFC environments and evacuation capabilities carried out via drones are being developed. New critical care capabilities and diagnostic tools to help guide triage and management of casualties will need to be validated to avoid increased mortality on future battlefields.

Among the injury mechanisms that are likely to cause increased mortality in future conflicts is chest trauma complicated by ARDS.[5,6] Data from the USAISR Joint Theater Trauma Registry from 2003-2011 during Operations Enduring Freedom and Iraqi Freedom (OEF/OIF) showed that among the 6,030 thoracic injuries, pneumothorax and pulmonary contusions were the most common (51.8% and 50.2%, respectively).[5] Pulmonary contusion (PC) is one of the common consequences of thoracic trauma and may lead to early-onset ARDS.[5,7,8] PC is an independent risk factor for pneumonia and ARDS and carries a 10%–25% mortality, as well as the possibility of significant long-term disability.[9,10]

Diagnosis of injury severity following PC and during early ARDS is a challenging task especially for combat medics and Special Operations Forces (SOF). To ease diagnosis of injury severity for SOF, a novel metric, HMGB1 protein, has been identified as a mediator of ARDS and is expressed in blood following activation of damaged cells after chest trauma or smoke inhalation injury.[11–13] In this study, we investigate HMGB1 expression in systemic blood samples obtained from uninjured and injured animals that undergo ECLS and AE as part of a larger combat relevant study. This is the first report of HMGB1 analysis from a severely injured animal model, which mimics human combat injuries and subsequent AE with extracorporeal life support (ECLS). This marker may enable the SOF medic to grade injury severity and prognosis of possible multiorgan failure (MOF) at or near the point of injury or while in transit during evacuation. This first report aims to inform future efforts toward mobile laboratory capability, potentially down to a "lab-on-a-chip" for far-forward use by SOF. We aim to eventually provide the SOF medic with this vital tool to monitor injury severity and inflammatory reaction of patients during PFC scenarios.

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Although not yet standard of care, extracorporeal membrane oxygenation (ECMO) is already used to support severely injured ARDS patients during AE and for that reason was part of the model used in this study.[14–16] In this pilot study, we investigate the systemic expression of HMGB1 in a model of ARDS due to bilateral PC treated with fluids, pressors, and venovenous ECLS. We hypothesized that HMGB1 levels increase after trauma and during AE.

Methods

This study was approved by a local institutional animal care and use committee (Protocol No. BPTS 15-02) and carried out in compliance with the Animal Welfare Act, principles of the "Guide for the Care and Use of Laboratory Animals," and all local, state, and federal guidelines for the ethical use of animals.

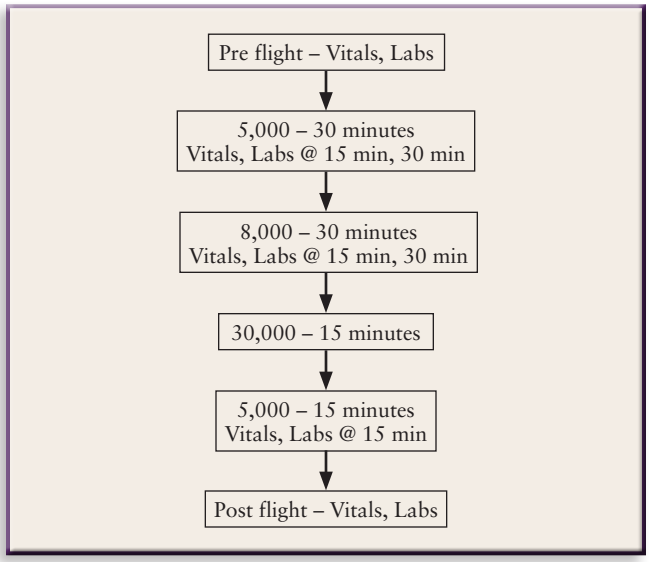
Experimental Procedures

Anesthetized, female Yorkshire pigs (N = 15, 53.8 ± 1.4kg) received arterial and venous catheters, tracheostomy, and Foley catheter placement. After baseline (BL) measurements, animals were cannulated and venovenous ECLS was initiated (CardioHelp; Maquet Gmbh, Gettinge Group, Rastatt, Germany) via Avalon 23Fr dual-lumen catheter (Getinge Group, Rastatt, Germany). Blood flow was 1.2–2.2L/min, and sweep gas flow ranged from 4 to 8L/min. Continuous heparinization was started at cannulation and titrated to 30%–50% higher than baseline ACT levels, as standard anticoagulation during ECLS therapy. Animals were then transported via a standard NATO litter fitted with a next-generation medical equipment rail kit (MERK; Smeed Technologies, Cummings, GA) to an adjacent building housing the hypobaric chamber. The altitude simulation profile is depicted in Figure 1. The altitudes chosen for testing correspond to both long-distance transport in pressurized aircraft (8,000 ft) and potential altitudes of transportation by drone (5,000 ft). In addition, we carried out a 30,000-ft step to study a rapid decompression scenario (e.g., during an aircraft losing cabin pressure). Altitude exposure occurred in the same animals in healthy state on day 1 (to study effects of ECLS without injury present) and in the injured state on day 2 (assuming ECLS would be used to treat trauma victims. The uninjured day 1 data served as control data for the injured day 2 experiments. Injury consisted of bilateral pulmonary contusions using a modified captive-bolt stunner (Model ML; Karl Schermer, Packers Engineering, Omaha, NE) with immediate chest-tube placement as previously described.[7,17,18] Heparin administration for systemic ECLS anticoagulation was discontinued ~8 hours prior to injury; thus, from midnight of day 1 and until the end of procedures on day 2, the animals received heparin-free ECLS for trauma because heparin would be contraindicated during trauma/hemorrhage.

Laboratory Measurements

Arterial blood samples were collected into EDTA blood-collection vacutainers (Becton, Dickinson and Company). On day 1, blood was collected at the following time points: BL, post-ECLS (PE), sea level, 5,000 ft, 8,000 ft, 30,000 ft, postflight, and 12 hours post-PE. On day 2, blood was collected at the following time points: preinjury, postinjury (PI), sea level, 5,000 ft, 8,000 ft, 30,000 ft, and postflight. Samples were centrifuged at 3000 rpm for 10 min at 4°C; plasma was stored at –80°C until analysis. Enzyme-linked immunosorbent assay (ELISA) was used to measure HMGB1 (ST51011; IBL International) in the blood at each time point. Plasma-free hemoglobin (pfHb) was measured via the direct spectrophotometric method as previously described.[19] Plasma total protein concentration (PTPC) was measured with use of the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

FIGURE 1 *Altitude exposure profile of each day's experiment.*



Histological Lung Injury Severity Assessment and Immunohistochemistry

For postmortem injury severity assessment, 1.5 × 1.5-cm lung samples were excised from both lungs. Samples were fixed in 10% normal buffered formalin for 48 hours, processed, embedded in paraffin, and then cut into 4µm sections. Slides were deparaffinized in histological grade xylene and dehydrated through graded alcohols to water, followed by staining with hematoxylin and eosin (H&E) and immunohistochemistry (IHC). Histological images were obtained with ×100 and ×200 magnification using an Axioskop microscope (Zeiss, Oberkochen, Germany). Diffuse alveolar damage (DAD) scores were the sum of the individual scores for fibrosis (%), alveolar interstitial fibrosis (IF), alveolar space (AS), alveolar protein aggregation (PA) and type II epithelial cell proliferation (EC), each on a scale of 0–4.[20–22]

IHC was performed to analyze HMGB1 and Toll-like receptor 4 (TLR4) expression in lung tissue. Following fixation in 10% normal buffered formalin (NBF), tissue was incubated with primary antibodies for HMGB1 (1:150, ab18256; Abcam Inc.) with standard IHC avidin-biotin-peroxidase complex technique (Elite ABC kits, Catalog No. PK-6100; Vector Laboratories, Burlingame, CA) with 3-diaminobenzidine tetrahydrochloride (DAB).

Statistical Methods

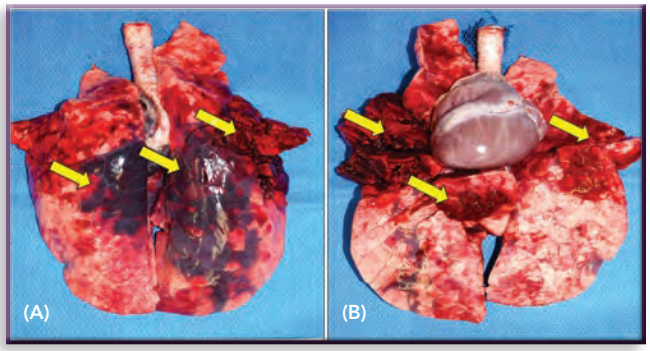
Statistics were performed using SAS version 9.4 (Cary, NC). All tests were two-sided with an $\alpha = .05$ for significance. First a Shapiro-Wilk test was conducted to test the distribution of the data for normality. If skewed, the data were then log transformed or the nonparametric version of the test was used. Data were analyzed using a one-way mixed model with repeated measures and a Dunnett adjustment to test for significant change from baseline. Group differences were examined using a two-way mixed model with repeated measures and a Tukey adjustment. All data are expressed as means ± standard error of the mean.

Results

Of 15 animals that entered the study, all completed day 1 (control conditions). Six animals died after PC but before flight on day 2: two died from suspected myocardial infarction following injury and four died from post-PC cardiac contusion and nonresponsiveness to vasopressors and fluids, signifying the severity of the model.

On gross observation, severe lung laceration, damage to major blood vessels, and severe contusion resulted in bilateral lung damage (Figure 2). Histological scoring of lung injury (diffuse alveolar damage [DAD]) is shown in Figure 3A (left lung) and 3B (right lung) for both left and right sides. Interstitial fibrosis (IF), alveolar space (AS), protein aggregation (PA), and type II epithelial cell (EC) proliferation scores were not different between right and left lungs (Figure 3C). The fibrosis percentage (15.7% ± 1.8% versus 15.0% ± 1.9%) and overall DAD scores (22.8 ± 2.4 versus 21.5 ± 2.2) were not different between lungs, an indication of identical injury to both lungs (Figure 3D).

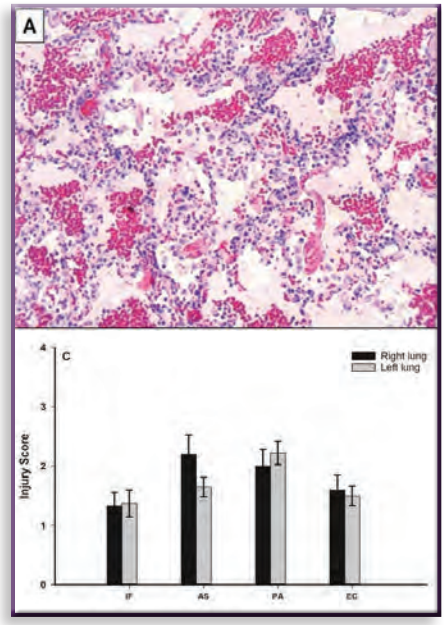
FIGURE 2 *Postmortem gross anatomy images of posterior lungs* **(A)** *and anterior lungs* **(B)** *after bilateral pulmonary contusion supported by ECLS. Arrows point to areas of consolidation as a result of trauma.*



Extracellular and paranuclear cytoplasmic immunochemical staining revealed HMGB1 and TLR4 in the lungs of all animals (Figure 4A, B). These mediators were primarily localized in the alveolar epithelial cells and neutrophils, monocytes, macrophages, and endothelium; however, there were no differences in the expression pattern/area or density of HMGB1 and TLR 4 in right versus left lungs.

Plasma HMGB1 on day 1 was transiently higher at arrival to the altitude chamber (D1 sea level time point) (Figure 5A). HMGB1 increased significantly after injury reaching highest values at 8,000 ft on day 2, after which levels decreased but remained elevated when compared to baseline (Figure 5A). pfHb decreased significantly on day 1 after ECLS initiation (PE time point), at 30,000 ft. and at the 12-hour time point (Figure 5B). On day 2, pfHb was higher at all time points but statistically significantly higher at 8,000 ft and postflight. Plasma total protein concentration (PTPC), a nonspecific measure of protein breakdown, was significantly decreased from day 1 sea level until the end of the experiment (Figure 5C), likely reflecting hemodilution due to resuscitation.

FIGURE 3 *DAD component scoring in right and left lungs. Representative images of the right* **(A)** *and left* **(B)** *lungs (hematoxylin and eosin stain; original magnification ×200).* **(C)** *Injury scores for alveolar interstitial fibrosis (IF), alveolar air space (AS), protein aggregate (PA), and expression of type II alveolar epithelial cell (EC).* **(D)** *Percentage of tissue fibrosis and DAD score. Values are means ± standard error.*



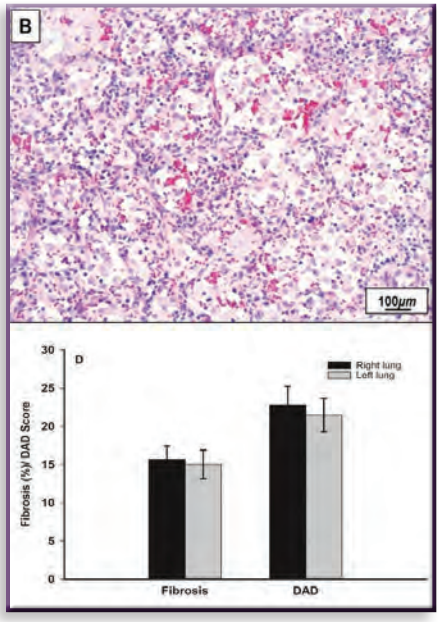
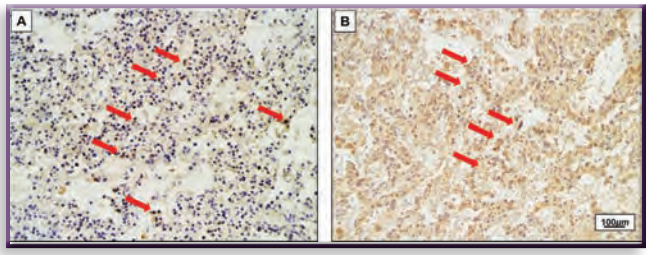


FIGURE 4 *Postmortem expression of (arrows) HMGB1* **(A)** *and TLR4* **(B)** *after bilateral pulmonary contusion.*



Discussion

This is the first report on the systemic expression of HMGB1 using a model of combat-relevant injury and AE managed with ECLS. We found that HMGB1 increased with trauma and remained elevated at subsequent stages of the study.

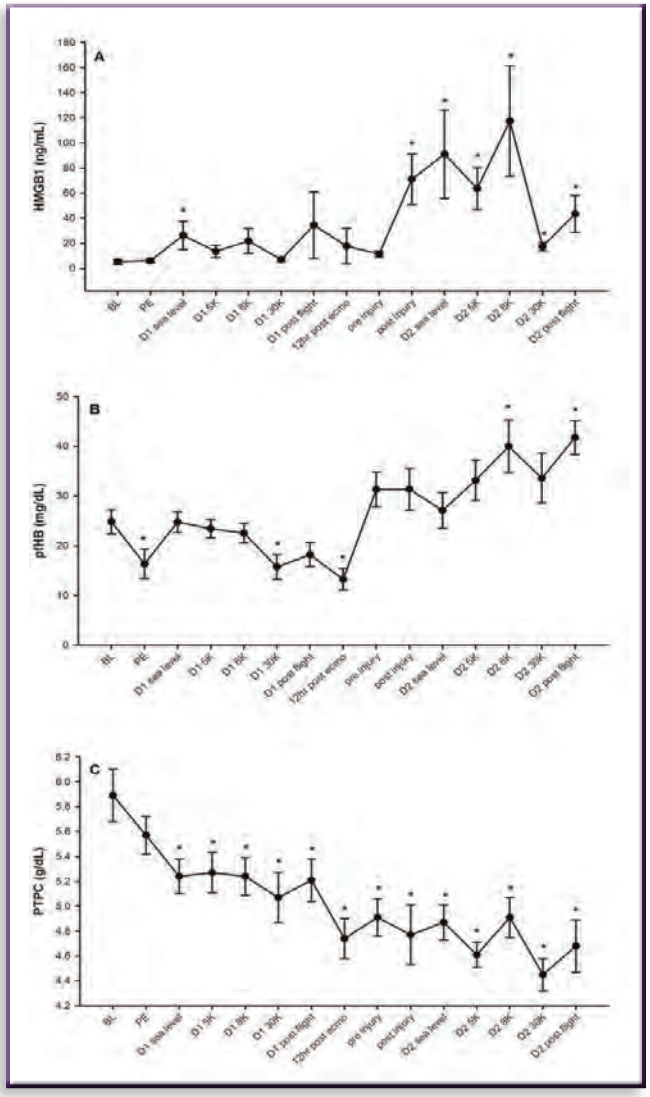


Figure 5 *Change in systemic extracellular HMGB1* **(A)**, *pfHb* **(B)**, *and PTPC* **(C)** *in a bilateral pulmonary contusion treated with ECLS at ground level and high altitude during en route care. Values are means ± standard error.* *P < .05 *determined by two-way analysis of variance with repeated measures. Baseline vs each time point.*

Injuries that occur in austere environments, particularly in the combat setting, require rapid assessment by medics and SOF and early evacuation with en route critical care support.[1,2] The US Air Force Critical Care Air Transport Teams (CCATTs) tasked with AE must perform advanced patient care and monitoring procedures in poorly lit conditions, with significant noise and vibration and marked barometric pressure changes. Mission duration is often extended, with the average mission from Iraq to Germany lasting approximately 6–8 hours.[23,24] These difficult evacuation conditions are likely to be worse during PFC future conflicts, creating significant challenges in point-of-injury care, diagnosis, and management of combat injuries by medics. These challenges will impact SOF even more, and new tools to assess the presence of injury, and its severity, available from lab-on-a-chip approaches would enhance the medics' ability to diagnose and triage casualties.

In current combat casualty care situations, advanced decision-support/ diagnostic tools are not commonly used. En route care diagnostic protocols are based on standard vital signs (e.g., blood pressure, blood gas, electrocardiography, mechanical ventilation information), which have proved to be of limited value in detecting the need to implement an intervention. In this pilot study, we extended the investigation of systemically released HMGB1, pfHb, and PTPC as injury acuity markers during high-altitude en route care with ECLS initiation to mimic combat-relevant transport. These acuity indices have not been validated in clinically relevant studies before because they are difficult to measure, are costly, and require invasive blood draws. Yet their utility in trauma/inflammation is evident.[11–13] Future casualty care during PFC and evacuation is likely to rely more heavily on fully or semiautonomous casualty evacuation platforms. These platforms will require real-time or near-real-time monitoring tools, such as levels of injury severity and inflammatory biomarkers, all made available to providers on the ground, potentially guiding changes in therapy. In this instance, the markers of injury severity will empower the SOF medic to recognize that someone is injured severely enough to require attention even if not visibly traumatized, an aspect of triage, as well as allow for monitoring of deterioration or improvement in condition based on continued expression or decrease in marker levels, respectively.

Following trauma and/or pathogen exposure, a variety of injury severity markers—damage-associated molecular patterns (DAMPs), homeostasis-altering molecular processes (HAMPs), pattern-recognition receptors (PRRs), and other molecules—have been recognized as mediators of the host response to injury.[25–27] Among these, HMGB1 is a nuclear protein, acting as a chromatin-binding cofactor that bends DNA and promotes access to transcriptional protein assemblies on specific DNA targets.[28–30] HMGB1 also functions as an extracellular signaling molecule—a damage-associated molecular pattern (DAMP). The relevance of HMGB1 as a therapeutic target and potential biomarker has been demonstrated by measuring it in plasma and tissue in numerous human diseases, including neurodegenerative disease, lung injury and disease, cancer, cardiac and vascular disease or injury, trauma ischemia–reperfusion injury, infection, and kidney- and liver-related disease.[25,31–34] Also, HMGB1 can signal disease progression in ARDS;[35,36] during inflammation,[37–39] cell proliferation,[40,41] or organ or tissue regeneration;[39,42] is a strong indicator of tumor or tumor-like cell development;[43,44] and correlates closely with sepsis and MOF.[11,12,45–47] According to Gardella et al., 65% of HMGB1 is confined to the nucleus in resting monocytes, but only 26% of HMGB1 is nuclear and 74% appears associated with cytoplasmic organelles in LPS-stimulated monocytes.[48] In activated monocytes, the transfer of HMGB1 from the nucleus to the cytoplasm is mediated by hyperacetylation.[49] Additionally, several types of inflammatory signals initiate export of nuclear HMGB1 to the cytoplasm and culminate in extracellular secretion.[46,50–52] Nuclear export has been shown to be regulated by oxidation, ADP-ribosylation, phosphorylation, secondary messengers (reactive oxygen species, calcium, nitric oxide), and both acetylation and methylation.[53,54] Although we did not measure markers of oxidative stress, it is possible that the exposure to high altitude can generate reactive oxygen species and modify calcium and nitric oxide levels in the high-altitude environment and in the presence of injury. Also, acetylation and methylation are major posttranslational DNA modification processes in the cell with many effects on the protein level and metabolome level.[54] Aigi et al. demonstrated that due to altitude exposure, HMGB1 increased in patients with congestive heart failure.[55] Studies showed that oxidative stress can promote the proinflammatory effects of HMGB1. The

increase in HMGB1 documented in our study adds to the body of work and collectively justifies its utilization in trauma care. Although there is currently insufficient information regarding the acute clinical implications of increased HMGB1 levels, the increased presence of this marker in systemic blood in our study suggests its potential utility as a predictor of multiorgan failure and death. Not only was the latter directly assessed in this study, but also future work will interrogate the association of HMGB1 increases with outcomes and end organ damage.

To achieve these goals, measurement of HMGB1 and other analytes in plasma/serum at point of injury will require development of new chip-based technology. The current ELISA method is very well established but would not be feasible in the field. Lateral flow immunochromatographic assay is one promising way to measure bedside markers.[56] However, it may have limited accuracy. Nanotechnology-based solutions are being developed to measure HMGB1 based on DNA nano-objects binding HMGB1, which could soon permit transition to point-of-need measurement.[57] Other microfluidics-based "lab-on-a-chip" solutions have been developed and may provide a pathway for cellphone-based analysis of injury indices.[58] All of these approaches would enhance the SOF medic's ability to diagnose, triage, and intervene in severely injured combat casualties.

We also studied pfHb, which has been identified as an independent predictor of mortality in patients receiving ECLS.[59] Circulating pfHb resulting from mechanically induced hemolysis and insufficient haptoglobin/hemopexin may promote thrombosis within the ECLS circuit.[60] Excess levels of pfHb cause Hb-mediated NO scavenging.[61] The subsequent depletion of NO can lead to increased systemic and pulmonary vascular resistance, increased thrombin formation, fibrin deposition, platelet aggregation, organ dysfunction, and increased mortality rate.[62–64] In this study, pfHb levels were lower than BL on day 1 but increased after injury at 8,000 ft and after descent on day 2 (Figure 5B). In our study, pfHb remained below 2 × BL (40mg/dL), thus below 50mg/dL, which was identified by Omar et al. as the harbinger of death 24 hours post-ECLS.[59] Longer duration of postinjury observation on ECLS needs to be explored to understand the utility of pfHb as a prognostic factor during prolonged use of extracorporeal technologies and a potential additional metric of injury severity and outcome during combat.

Postmortem lung histology showed no difference in injury scores between left and right lungs attesting to the reproducibility of the pulmonary contusion (Figure 4C). The bilateral PC model was used as has been previously reported.[7,17,18] It is characterized by transient rapid desaturation immediately postcontusion, with the development of true shunt and hemodynamic depression.[7,17,18] In this study, 50% of the animals required continuous administration of pressors and fluids to support life. The severity and clinical relevance of the model are also shown by six nonsurvivors dying immediately or soon after chest trauma. In these nonsurvivors, higher levels of HMGB1 and pfHb were observed (data not shown). In the future, we plan to the extend the duration of care to 72 hours after injury to study associations with mortality.

Conclusion

HMGB1 showed sustained elevation after trauma and during altitude transport and may provide a useful monitoring capability during PFC and en route care. Rapid point of injury lab-on-a-chip assays and anti-HMGB1 therapy are potential diagnostic and treatment modalities that can be useful in the management of patients with chest trauma and ARDS and may enhance the decision-making capability of SOF medics.

Author Contributions

All authors participated in study design, protocol writing, analysis of data and manuscript writing and critical revisions.

Disclosures/Conflicts of Interest/Funding

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