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Hypobaric Hypoxia Exacerbates the Neuroinflammatory Response to Traumatic Brain Injury

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Abstract

Objective—To determine the inflammatory effects of time-dependent exposure to the hypobaric environment of simulated aeromedical evacuation following traumatic brain injury (TBI).

Methods—Mice were subjected to a blunt TBI or sham injury. Righting reflex response (RRR) time was assessed as an indicator of neurologic recovery. Three or 24 h (Early and Delayed groups, respectively) after TBI, mice were exposed to hypobaric flight conditions (Fly) or ground-level control (No Fly) for 5 h. Arterial blood gas samples were obtained from all groups during simulated flight. Serum and cortical brain samples were analyzed for inflammatory cytokines after flight. Neuron specific enolase (NSE) was measured as a serum biomarker of TBI severity.

Results—TBI resulted in prolonged RRR time compared with sham injury. After TBI alone, serum levels of interleukin-6 (IL-6) and keratinocyte-derived chemokine (KC) were increased by 6 h post-injury. Simulated flight significantly reduced arterial oxygen saturation levels in the Fly group. Post-injury altitude exposure increased cerebral levels of IL-6 and macrophage inflammatory protein-1 α (MIP-1 α), as well as serum NSE in the Early but not Delayed Flight group compared to ground-level controls.

Conclusions—The hypobaric environment of aero-medical evacuation results in significant hypoxia. Early, but not delayed, exposure to a hypobaric environment following TBI increases the neuroinflammatory response to injury and the severity of secondary brain injury. Optimization of the post-injury time to fly using serum cytokine and biomarker levels may reduce the potential secondary cerebral injury induced by aeromedical evacuation.

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Keywords

traumatic brain injury; inflammation; neuroinflammation; aeromedical evacuation; hypo-baric; hypoxia

INTRODUCTION

Clinical outcome following traumatic brain injury (TBI) is dependent on the neurological damage that evolves after the initial trauma. Whereas primary brain injury refers to the damage caused at the moment of traumatic impact, secondary brain injury is the result of progressive brain damage from the accumulation of cellular and vasogenic edema. A principal goal of care following head injury is to prevent the development of secondary brain injury from insults such as hypoxia and hypotension that significantly increase the risks of post-traumatic morbidity and mortality [1]. Post-traumatic neuroinflammatory responses play an increasingly recognized role in the development of secondary brain injury by contributing to neural damage induced by the recruitment of circulating neutrophils and the activation of local astrocytes. Cerebral and systemic release of cytokines and chemokines modulate the post-injury inflammatory response and can be correlated with clinical prognosis.

The modern United States Air Force aeromedical evacuation (AE) system has evolved into a worldwide patient airlift capable of providing rapid and safe transport of military patients to specialized medical facilities. Current use of advanced *en route* care has enabled transport of injured soldiers to higher echelon hospitals more rapidly than in any previous military conflict [2, 3]. The AE process, including exposure to a hypobaric environment and timing of post-injury air transport, may affect injury outcome.

Potential effects of AE on systemic and cerebral inflammatory responses after TBI have not been studied. In the present study, we used a murine model of moderate TBI to evaluate the effects of simulated AE on the local brain and systemic inflammatory responses and how these effects changed with increased time between initial injury and simulated AE. Our results indicate that monitoring of patient immunologic status may help optimize the appropriate time for AE in patients suffering TBI.

METHODS

Animals

Male C57/BL6 mice weighing 22–29 g were purchased from Charles River Laboratories (Wilmington, MA), fed standard laboratory diet and water *ad libitum* and acclimated for 2–3 wk in a climate-controlled room with a 12-h light-dark cycle. Experiments were approved by the Institutional Animal Care and Use Committees of the University of Cincinnati and the United States Air Force facilities at Brooks City-Base, Texas.

Head Injury Model

Blunt traumatic brain injury was induced utilizing a 415 g cylindrical weight-drop device with a 1 cm wide striking surface placed within a plastic guide tube. Mice were anesthetized for 2 min with 2% isoflurane in 100% oxygen at 1 L/min. Animals were then placed in a prone position on a platform below the head injury device so that the central region between coronal and lambdoid sutures was centered beneath the guide tube [4]. Head injury was delivered by dropping the weight from a 1.5 cm height. Rebound impact was prevented by securing the weight rod immediately after initial impact. Sham-injury mice were anesthetized and positioned in the same manner as injured mice but were not subjected to head injury.

Following traumatic weight impact, head-injured and sham mice underwent neurological evaluation using the righting reflex response (RRR). The RRR is defined as the animal's ability to right itself to a prone position three times consecutively after being placed supine immediately after injury [5]. Time to achieve righting was recorded for each animal. Occurrences of post-injury apnea, convulsions, and visible limb paralysis were also noted. A group of animals were subjected to TBI or sham injury and were sacrificed at intervals post-injury (1, 3, 6, 12, 18, 24 h, $n = 5$ each group) to characterize the time course of inflammation for this TBI model.

Simulated Aeromedical Evacuation

All simulated altitude experiments were performed in a hypobaric chamber at Brooks City-Base in San Antonio, TX operated by United States Air Force aerospace physiology personnel. The study protocol was designed to mimic a standard Critical Care Air Transport Team (CCATT) aeromedical evacuation flight. At 3 or 24 h after TBI or sham injury, animals were transported from the housing facility to the altitude chamber. Mice were then exposed to a simulated cabin altitude of 8800 feet for 5 h. Sham (No Fly) animals were transported to and from the chamber facility with the altitude-exposed animals (Fly), but remained at ground level (600 feet elevation) for the duration of the simulated flight (Fig. 1). Ambient temperature was maintained at 72°F for all animals at simulated altitude and ground level.

Serum and Tissue Analysis

Prior to descent from 8800 feet altitude, groups of head-injured and uninjured mice both in and outside the hypobaric chamber were assessed for oxygenation levels ($n = 5$ each group). Following light sedation with 0.3 mg pentobarbital, an arterial blood sample was obtained by transdiaphragmatic cardiac puncture and immediately analyzed using an iSTAT (Heska Corp., Loveland, CO).

Animals were sacrificed at intervals (1, 6, 12, and 24 h, $n = 5$ each group) following simulated AE. Blood was obtained by cardiac puncture and the brain was excised by immediate *post-mortem* craniectomy. Serum and left cortical brain samples were analyzed at each time point for levels of inflammatory cytokines using customized multiplex enzyme-linked immunosorbent assay (ELISA) technology (Quansys, Logan, UT). Fifteen cytokines and chemokines were evaluated in the ELISA analysis, including GM-CSF, IFN γ , IL-1 β ,

IL-4, IL-6, IL-10, IL-12p70, IL-17, IP-10, KC, MCP-1, MDC, MIP-1 α , RANTES, and TNF α . Cortical samples were homogenized in 1 mL of phosphate buffered saline containing a complete tab protease inhibitor cocktail (Roche, Indianapolis, IN). Supernatants were centrifuged three times at 12,000 g for 15 min each. Serum was extracted from blood samples by centrifugation at 8000 rpm for 10 min in serum separator tubes. Serum was stored at -80 °C until analysis. The multiplex ELISA was performed per manufacturer instructions. Cerebral cytokine levels were normalized to cortical protein content using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Neuron specific enolase (NSE) was measured as a serum indicator of head injury severity using a commercially available ELISA kit (Immuno-Biological Laboratories, Inc., Minneapolis, MN).

Statistical analysis was performed by two-tailed *t*-test or one-way ANOVA with Tukey's *post-hoc* analysis for multiple comparisons. Spearman rank order was used to test correlations between quantitative variables for significance using SigmaStat 3.5 (Systat, Chicago, IL). Data are reported as mean $\pm$ SEM. *P* <0.05 was considered significant.

RESULTS

Decreased Neurological Response and Increased Markers of Systemic Inflammation After TBI

In order to validate our weight-drop model of TBI, we determined the effect of anesthesia (sham) or head injury on RRR. Sham injured mice demonstrated minimal loss of the RRR following anesthesia alone. By contrast, head injury induced significantly longer loss of the RRR (Fig. 2). Apnea, convulsions, and limb paralysis were not noted in the TBI group. Induction of TBI resulted in 11% mortality, consistent with previous murine blunt head injury models [4]. All deaths occurred immediately after weight-drop impact without post-injury neurological recovery.

Following TBI, serum pro-inflammatory cytokine levels were measured over a 24-h time course. TBI induced significant increases in serum IL-6 and KC compared with sham-injured mice 6 h after TBI (Fig. 3A, B). No significant differences were found in cerebral levels of IL-6 or MIP-1 α between sham and TBI groups at any time point (Fig. 3C, D). No differences were found in cerebral or serum levels of the other cytokines and chemokines analyzed.

Simulated AE Causes Hypoxia in Injured and Uninjured Animals

To determine the extent to which simulated flight induced hypoxia in sham or TBI injured mice, oxygen saturation levels were measured after 5 h of simulated AE (Fly) or ground-level control (No Fly). Oxygen saturation levels were significantly lower in animals exposed to hypobaric conditions for 5 h than those remaining at ground level (approximately 600 feet). Similar decreases in arterial oxygen saturation were found in both sham and TBI groups exposed to simulated AE (Fig. 4). Anesthesia administered to obtain the blood gas sample did not significantly alter $p\text{CO}_2$ levels, indicating that sedation did not contribute to detected hypoxia.

Early But Not Delayed Simulated AE Augments the Neuroinflammatory Response to TBI

To determine whether simulated AE had an effect on systemic inflammation induced by TBI, serum samples were obtained 1, 6, 12, and 24 h after control or altitude exposure in mice exposed to sham injury or TBI. No

In order to evaluate the effect of simulated AE on the neuroinflammatory response, left hemispheric cortical samples were analyzed for cytokine and chemokine expression 1, 6, 12, and 24 h after simulated AE (Fly) or ground-level control (No Fly). No differences were observed in cortical cytokine levels 1, 6, or 12 h after flight. However, mice receiving TBI 3 hours prior to simulated AE (Early Flight) had increased cerebral levels of IL-6 and MIP-1 α compared to ground-level controls (No Fly) at 24 h post-flight (Fig. 5A). There were no differences in brain IL-6 or MIP-1 α levels in the Delayed Flight group (Fig. 5B). These data suggest that exposure to hypobaric hypoxia shortly after TBI is associated with increased cerebral cytokine expression.

Simulated AE Increases Secondary Brain Injury after TBI

To determine if the augmented neuroinflammatory response observed in TBI/Fly groups resulted in increased brain injury, serum NSE levels were measured 1, 6, 12, and 24 h after simulated AE. Mice receiving TBI 3 h prior to simulated AE showed a significant increase in NSE 24 h post-flight compared with the No Fly group (54.8 ± 11.6 μ g/L *versus* 13.2 ± 2.5 μ g/L, TBI/Fly *versus* TBI/No Fly, $P < 0.001$) (Fig. 6). In addition, 24 h after simulated AE, animals subjected to TBI and early post-injury flight had significantly higher levels of serum NSE than those undergoing delayed post-injury flight (54.8 ± 11.6 μ g/L *versus* 7.5 ± 1.5 μ g/L, 3 h TBI/Fly *versus* 24 h TBI/Fly, $P < 0.01$). Furthermore, in mice subjected to TBI 3 h prior to simulated AE, serum NSE levels significantly correlated with cerebral IL-6 levels throughout the post-flight time course ($r = 0.50$, $P = 0.02$).

DISCUSSION

The current study is the first to examine the effects of hypobaric hypoxia, as occurs during AE, on the local brain and systemic inflammatory response. We established a reproducible model of blunt head injury and characterized the time course of the systemic and cerebral inflammatory responses. Using this model of TBI, we investigated the effects of hypobaric hypoxia associated with current AE protocols on the inflammatory response to blunt head injury. Our data provide strong evidence suggesting early exposure of mice with TBI to hypobaric hypoxia (within 3 h) results in an augmented neuroinflammatory response and increased secondary brain injury, whereas delayed exposure to hypobaric hypoxia (24 h after TBI) has no harmful effects.

Exposure to an in-cabin pressure corresponding to an altitude of up to 9,000 feet occurs routinely during military AE as well as some commercial airline flights. Although the development of an 8000 foot aircraft structural design criteria by the Federal Aviation Administration has been deemed adequate for healthy individuals, patients with cardiopulmonary compromise are at risk for the significant hypoxemia under these conditions [6]. Healthy volunteers acutely exposed to 7872 feet (2400 m) altitude had

significantly reduced oxygen saturation levels to 93% [7]. Similarly, airline crew members continuously monitored by pulse oximetry during commercial flights demonstrated a mean peripheral oxygen saturation of 88.6% at cruising altitude [8]. Our results demonstrate a comparable effect of hypobaric hypoxia, with arterial oxygen saturations of 87% at 8800 feet (2440 m) in both sham and head-injured mice. Although there was no oxygen saturation difference between the TBI and sham-injured mice, hypoxemia following head injury has been shown to aggravate secondary brain injury [9, 10]. Additional physiologic and experimental parameters that may be altered by exposure to a hypobaric environment and potentially affect TBI-induced neuroinflammation and head injury outcome include blood pressure and length of time exposed to hypobaric conditions. In this initial series of experiments, we chose not to measure blood pressure during simulated flight because of the inflammatory impact of invasive blood pressure measurement and lack of satisfactory noninvasive blood pressure monitoring capabilities in a murine model. Our flight time was initially based on the average AE flight time between in- and out-of-theater hospitals in the current Operation Iraqi Freedom/Operation Enduring Freedom conflicts. Certainly the physiologic and inflammatory effects of varied simulated flight time remain to be elucidated.

A model of moderate blunt weight-drop impact was chosen for this study to mimic the prevalent concussive brain injury associated with blast effect seen in the casualties of modern military conflict. Similar centralized weight-drop head injury models in mice have demonstrated transient neurobehavioral depression, short-term cerebral edema associated with blood-brain barrier permeability, and persistent learning and memory deficits [4, 11]. The parallel systemic and cerebral inflammatory responses to blunt TBI, however, have not been comprehensively studied for this model. Our data demonstrate concordant timelines of pro-inflammatory cytokine release in the serum and injured cerebral cortex, with peak cytokine levels at 6 h post-injury. It is notable, however, that the human post-TBI neuroinflammatory response can last weeks, whereas in animals cytokine production peaks within hours and is undetectable within 7 d [12]. Characterization of the post-TBI time course of systemic inflammation may be used to help determine immune status at the time of a potential post-traumatic “second hit” stimulus.

Evaluation of the post-traumatic systemic inflammatory response demonstrated significant elevation of IL-6 and KC levels. Elevation of both cytokines was evident as early as 1 h, peaked at 6 h, and returned to baseline levels within 24 h post-injury. Human studies have similarly reported increased IL-6 and IL-8 levels in both the serum and cerebrospinal fluid (CSF) following moderate and severe TBI [13–24]. Comparisons of post-TBI CSF and serum cytokine levels have routinely demonstrated higher IL-6 and IL-8 in the CSF than in plasma, suggesting that post-TBI cytokine disseminates from the brain into the peripheral circulation [17, 18, 20, 21]. Although the use of CSF IL-6 levels as an individual prognostic indicator of clinical outcome has yielded inconsistent results, sustained elevation of plasma IL-6 after TBI predicts a poor prognosis and increased risk of infectious complications [15, 23, 25]. Similarly, survival following severe TBI has been associated with lower plasma levels of IL-8 [15, 24].

Cerebral cytokine analysis following blunt traumatic brain injury in the present study demonstrated trends toward significantly increased levels of cortical IL-6 but not MIP-1 α . These results are similar to those of previous rodent TBI models that have demonstrated peak IL-6 levels at 4 to 8 h post-TBI [12, 26–28]. IL-6 has also garnered interest as a marker of neuroinflammation in the CSF of head injured patients, as previously mentioned. In addition to generating a systemic acute phase response, IL-6 release from the brain post-TBI promotes local post-traumatic wound healing [29, 30].

The neuroinflammatory response to TBI was exacerbated by early post-injury exposure to the hypobaric, hypoxic environment of a simulated AE flight. To our knowledge, this is the first study to investigate the post-injury effects of altitude and the impact of the timing of the initial post-injury flight. Levels of IL-6 and MIP-1 α were increased by 24 h post-flight only in the early flight group. Although previous studies have also demonstrated elevated plasma levels of IL-6 in humans acutely exposed to high altitudes, there have been no *in vivo* studies to date on the effects of altitude on MIP-1 α [31–33].

Neuron specific enolase has been shown to correlate with both primary brain injury severity and quantitatively assist in the prediction of long term neuropsychologic dysfunction and mortality following traumatic brain injury [34, 35]. NSE is a glycolytic enzyme predominantly found in neuronal cytoplasm that leaks into the extracellular compartment and bloodstream only after structural damage of both the neuronal cell and blood brain barrier [34, 36, 37]. In this study, we demonstrate that early post-injury exposure to the hypobaric conditions of AE causes an increase in the serum levels of NSE. Although numerous clinical studies have demonstrated that higher serum biomarker increases correlate with worsened neurologic outcomes, our data demonstrate a direct correlation between serum biomarker levels and a defined second hit phenomenon following TBI. Furthermore, this is the first study to correlate post-injury serum NSE levels to the neuroinflammatory response.

While initial serum biomarker concentrations represent the severity of the primary brain injury and associated immediate cell death and blood brain barrier destruction, peak concentrations and delayed bio-marker elevation suggest ongoing cell death as a part of secondary brain injury [13]. It is this secondary brain injury, such as that occurring with post-injury hypoxia or hypotension, which more strongly relates to outcome. The current study demonstrates that neuroinflammation is augmented by early, but not delayed, post-injury simulated AE, and is also associated with elevated levels of serum NSE, suggestive of increased brain injury.

Taken together, our data suggest that early, but not delayed, post-TBI altitude exposure intensifies the neuroinflammatory reaction to the initial head trauma. The clinical challenge facing AE teams is to accurately determine when the brain is “fit to fly.” In our model, early *versus* delayed evacuation was defined by time post-injury alone. Head-injured patients, however, have more individual neurologic and inflammatory responses to TBI. Analysis of our data demonstrates that periodic evaluation of the post-traumatic systemic inflammatory profile for IL-6 and IL-8 (KC) may provide surrogate markers for the ongoing neuroinflammatory response. Awaiting normalization of these inflammatory cytokines may

allow medical transport personnel to determine the optimal post-injury time to fly after resolution of the initial immune insult. In addition, NSE may serve as a reliable serum biomarker to assess the progression of secondary brain injury induced by modulating the post-injury time to fly.

CONCLUSIONS

Our model of blunt head injury results in a significant systemic as well as cerebral inflammatory response. The hypobaric environment of simulated AE causes significant hypoxia for both injured and uninjured animals. Hypobaric hypoxia may contribute to secondary brain injury by increasing post-TBI neuroinflammation. Early, but not delayed, exposure to a hypobaric environment following TBI increases the post-injury neuroinflammatory response and exacerbates the progression of secondary brain injury. Optimization of post-injury time-to-fly based on the systemic inflammatory profile may reduce the potential cerebral damage induced by AE.

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FIG. 1.

Time course of experimental protocol. Animals were initially randomized to TBI or sham injury and then randomized again to simulated aeromedical evacuation or ground level control. Mice were sacrificed at 1, 6, 12, or 24 h post-flight.

FIG. 2.
Righting reflex response recovery time following traumatic brain injury (TBI) or sham.
* $p < 0.001$. differences were found in serum cytokines between groups.

FIG. 3. Proinflammatory cytokine levels of IL-6 (A) and KC (B) in the serum, and IL-6 (C) and MIP-1 α (D) in the brain over the first 24 h following blunt head injury (filled square) or sham (open circle). * $P < 0.05$ versus sham.

FIG. 4.

Oxygen saturation levels from arterial blood gas samples taken from head-injured (TBI) or uninjured (Sham) animals at ground level (No Fly) or 8800 feet altitude in a hypobaric chamber (Fly). * $P < 0.05$ versus Uninjured No Fly; ** $P < 0.05$ versus TBI No Fly.

FIG. 5.

Cerebral levels of proinflammatory cytokines IL-6 (A) and MIP-1 α (B) at 24 h post-flight in the 3 and 24 h preflight injury groups. * $P < 0.05$ versus TBI No Fly.

FIG. 6.

Serum NSE levels post-flight in the Early and Delayed flight injury groups. * $P < 0.001$ versus TBI/No Fly, ** $P < 0.01$ versus Delayed flight TBI/Fly.