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HYPOBARIA OR HYPOXIA: WHICH INSULT MATTERS MOST TO THE INJURED BRAIN

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14. ABSTRACT
Traumatic brain injury (TBI)-related morbidity is caused by a primary insult occurring at the time of injury followed by secondary damage caused by hypoxia, excessive sympathetic drive, and uncontrolled inflammation. The military routinely utilizes aeromedical evacuation (AE) to transport wounded warriors to higher levels of care rapidly. The hypothesis is that both hypobaric & hypoxic conditions would contribute to more severe TBI-related secondary injury in a porcine model. Results show the hypobaric environment of AE worsens the systemic release of several neuroinflammatory cytokines and may contribute to secondary injury following TBI. The mitigation of hypoxemia does not improve this systemic inflammatory response to AE via supplemental oxygen or the strategy of Cabin Altitude Restriction. Further studies are warranted to analyze the systemic inflammatory response following TBI and delayed hypobaric exposure, representing delayed AE following traumatic injury.

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1.0. SUMMARY/DISCLAIMER

Regulatory Approvals: This study was reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee (23-01-09-02) and the United States Air Force (USAF) Medical Readiness Agency, USAF Animal Use Oversight & Compliance (AFOSR-2020-0004A)

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2.0. BACKGROUND

Traumatic brain injury (TBI) represents a significant source of morbidity and mortality in the acutely injured trauma patient. The natural course of TBI involves two injury phases, including a primary and secondary insult. The primary injury refers to the direct and immediate result of head trauma, causing damage to the brain parenchymal tissue, cerebral thrombosis or blood flow disruption, and alterations in neuronal metabolism.[1,2] The secondary injury represents delayed, non-mechanical damage to the brain tissue often resulting from physiologic insults associated with the acute phase of polytrauma, including hypotension, hypoxia, and profound systemic inflammation.[3,4] Given the inherent irreversibility of the primary injury associated with TBI, the central therapeutic goal in minimizing TBI-related morbidity is the mitigation of factors leading to secondary injury.[5]

Combat trauma victims in the theater of war and civilians traumatically injured in austere, rural, or remote environments often require aeromedical evacuation (AE) for prompt stabilization and definitive care. AE transport modalities include rotary-wing (helicopter) and fixed-wing (airplane) aircraft with variable capabilities with respect to speed, altitude, and cabin pressurization.[6–8] Previous literature demonstrates a relationship between hypoxemia at altitude and systemic inflammation.[9,10] While cabin pressurization is key to minimizing exposure to the hypobaric conditions of flight, such pressurization increases the cost of transport via reduction in aircraft fuel efficiency, prolonged transport times, and increased structural stress on the aircraft.[11,12]

The effect of the hypobaric and hypoxic environment inherent to AE on the development and worsening of secondary injury associated with TBI is not well-described. The aim of our study is to determine the extent to which conditions of hypobaria and hypoxia, either isolated or combined, associated with AE may affect secondary brain injury following TBI.

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3.0. METHODS

3.1 *Animal Model*
This study was reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee and the United States Air Force Medical Readiness Agency Office of Research Oversight and Compliance. The animal care program was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and in compliance with the National Research Council's 2011 Guide for the Care and Use of Laboratory Animals, as well as Department of Defense Instruction 3216.1. All experiments were consistent with the ARRIVE guidelines 2.0. **(SDC-1)**

The porcine model utilized in the study consisted of female Yorkshire pigs obtained from Isler Genetics (Prospect, OH). Following arrival, the models were acclimated in our animal facility for 48-72 hours before experimentation, either alone or in pairs and provided with food and water without restriction. The animals were fasted the night prior to study initiation to prevent aspiration during induction of anesthesia. Pigs were sedated with tiletamine hydrochloride (Telazol) and xylazine hydrochloride (both given 5 mg/kg intramuscularly; Henry Schein Animal Health, Dublin, OH) and placed in the prone position prior to orotracheal intubation. Pigs were maintained on a ventilator (731 Series, Zoll Medical, Chelmsford, MA), which compensates for changes in altitude required by the study protocol. Ventilator settings were manipulated to maintain normal pH and desired oxygenation saturation throughout the study period. Each pig underwent placement of a right-sided femoral vein central venous catheter, a right-sided subclavian vein central venous catheter, and a left femoral arterial line via cutdown technique under direct visualization.

3.2 *TBI and intracerebral monitor placement*
Sixty pigs underwent general anesthesia and mechanical ventilation prior to induction of TBI via controlled cortical injury (CCI) as previously described.[13] Briefly, following excision of scalp tissue overlaying the crown of the frontal bone via electrocautery, a right-sided 2cm craniotomy was made 16 millimeters (mm) anterior to the coronal suture and 12mm lateral to the sagittal suture leaving the underlaying dura intact. A 5.3 mm craniotomy was made on both the ipsilateral (1 centimeter (cm) anterior to 2cm craniotomy) and contralateral side of the skull for placement of intracranial monitors. CCI was then induced via a 15 mm impactor at 4 meters/second for 100 millisecond dwell time and 13mm depth of impact onto exposed dura and underlying parenchyma. Quad lumen bolts were placed into adjacent craniotomy sites. OxyLite tissue oxygenation probe and OxyFlo blood flow probe (Oxford Optronix, Milton, United Kingdom) were inserted into brain parenchyma via the two quad lumen bolts. Given the use of the craniotomy sites for probe placement and monitoring throughout the experiment, the craniotomy sites were not closed. Pigs in the sham TBI group underwent single 2cm craniotomy without CCI delivery, as well as two 5.3 mm craniotomies (as described above) for the placement of intracranial monitors.

3.2 *Altitude/Hypoxia Exposure*
Pigs were randomized to one of 6 groups prior to initiation of the study including TBI versus sham-TBI, ground-level vs. 12,000 feet (ft) exposure, and normoxia (100 percent (%) oxygen saturation (SpO_2)) vs. relative hypoxia (85% SpO_2). The treatment groups included (1) Sham TBI, Ground level, 100% fraction of inspired oxygen (FiO_2) (Sham-Ground-100; control), (2) TBI, Ground level, 100% FiO_2 (TBI-Ground-100; isolated TBI) (3) Sham TBI, Ground level, 85% FiO_2 (Sham-Ground-85; isolated hypoxia), (4) TBI, Ground level, 85% FiO_2 (TBI-Ground-85; hypoxia TBI), (5) TBI, 12,000 ft altitude, 100% FiO_2 (TBI-12,000-100,000), (6) TBI, 12,000ft altitude, 85% FiO_2 (TBI-12.000-85,000). In order to titrate hypobaric exposure, 4 additional groups included exposure to either 5,000 ft or 8,000 ft of altitude as well as normoxia (100% SpO_2) vs. relative hypoxia (90% SpO_2 for 8000 ft group, 95% SpO_2 for the 5000 ft group). These treatment groups included (7) TBI, 8,000ft altitude, 100% SpO_2 (TBI-8,000-100,000), (8)

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TBI, 8,000ft altitude, 90% SpO_2 (TBI-5,000-90,000), (9) TBI, 5,000 ft altitude, 100% SpO_2 (TBI-5,000-100,000), (10) TBI, 5,000ft altitude, 95% SpO_2 (TBI-5,000-95,000).

Following delivery of the TBI or sham-TBI, flight pigs underwent simulated aeromedical evacuation to 12,000/8,000/5,000 ft (dependent on treatment group) for a duration of 90 minutes, while ground pigs were not exposed to the hypobaric conditions of flight. Throughout the 90-minute flight, the FiO_2 of the mechanically ventilated pigs was manipulated to a goal SpO_2 of either 100% (normoxia) or 85/90/95% (relative hypoxia, dependent on treatment group) as measured by a tongue pulse oximetry sensor. Such manipulation was liberated following completion to simulated aeromedical evacuation allowing for normoxia >97%.

3.3 *Serum Analysis*

Whole blood was collected prior to TBI delivery (baseline), immediately following flight (90 minutes post-injury), and at the end of the observation period (6 hours post-injury). Whole blood was then aliquoted into serum separator tubes and centrifuged at 10,000 gravitational forces (g) for 10 minutes for isolation of serum. Serum was then analyzed for cytokines including interleukin 1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) via the Qplex Porcine Chemokine High Sensitivity enzyme linked immunosorbent assay (ELISA) according to manufacturer protocol (Quansys Bioscience, Logan, UT). Serum was also analyzed for Hypoxia Inducible Factor 1α (HIF-1α, MyBioSource, San Diego, CA), Ubiquitin C-terminal Hydrolase L1 (UCH-L1, MyBioSource, San Diego, CA), Amyloid A (MyBioSource, San Diego, CA), and Neuron-specific Enolase (NSE, MyBioSource, San Diego, CA) by ELISA according to manufacturer protocols.

3.4 *Histology and protein quantification*

After euthanasia, the skull cap was removed, and dura incised for the purposes of brain removal. Given difficulty with sectioning of fresh brain tissue, the organ was stored first in 10% neutral buffered formalin (Thermo Scientific, Waltham, MA) for a period of 48 hours. The brain was then sectioned into 5 mm coronal slices with subsequent isolation of the hippocampus and frontoparietal cortex (approximately 2 cm from anterior brain surface) from both the left (uninjured) and right (injured) side. Brains were then immersion-fixed at room temperature using 10% neutral buffered formalin (Thermo Scientific, Fremont, CA) and tissue was processed and embedded in paraffin for light microscopy.

Cerebral tissue was cut to 5 micrometer (μm) sections and placed on glass slides. Antigen retrieval was performed via heat induced epitope retrieval in 1× citrate buffer (Thermo Scientific, Fremont CA). Sections were blocked with ready to use Bloxall (Vector Labs, Newark CA) for 10 minutes followed by avidin and biotin blocking solutions (Biocare Medical, Pacheco, CA) for 10 minutes each, respectively. Slides were washed (×2) with 1× tris buffered saline with tween (TBS-T) buffer (Thermo Scientific, Fremont, CA) for 5 minutes each. Sections were then blocked with serum in phosphate-buffered saline solution for 1 hour at room temperature.
For p-tau staining, sections were incubated overnight at 4°C with a primary rabbit polyclonal antibody to anti-tau (1:1000, celciusT231; Abcam, Waltham, MA) in a commercially available diluent (Thermo Scientific, Fremont CA). Sections were washed ×2 and incubated at room temperature with a biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Labs, Newark CA) in a commercially available diluent (Thermo Scientific, Fremont CA) for 30 minutes). Sections were washed ×2 and incubated at room temperature with a ready to use horseradish peroxidase streptavidin solution (Vector Labs, Newark CA) for 30 minutes. Lastly, sections were developed with 3,3' Diaminobenzidine (DAB) (Vector Labs, Newark CA), dehydrated to xylene, and coverslipped using Permount mounting medium (Fisher, Hampton, NH).

For glial fibrillary acidic protein (GFAP) staining, sections were then blocked with serum in phosphate-

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buffered saline solution for 1 hour at room temperature. Next, sections were incubated overnight at 4 degrees celsius (°C) with a primary rabbit polyclonal antibody to anti-GFAP (1:2000; Abcam, Waltham, MA) in a commercially available diluent (Thermo Scientific, Fremont CA). Sections were washed ×2 and incubated at room temperature with a biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Labs, Newark CA) in a commercially available diluent (Thermo Scientific, Fremont CA) for 30 minutes). Sections were washed ×2 and incubated at room temperature with a ready to use horseradish peroxidase streptavidin solution (Vector Labs, Newark CA) for 30 minutes. Lastly, sections were developed with 3,3' DAB (Vector Labs, Newark CA), dehydrated to xylene, and coverslipped using Permount mounting medium (Fisher, Hampton, NH).

Finally, a total of 4-6 random images or "Regions of Interest" were captured utilizing the Cytation C10 microscope and corresponding Gen5 3.12 software (Agilent Technologies, Santa Clara, CA). Regions of interest were captured at 20x magnification and analyzed utilizing the program's cell count and staining intensity measurement capabilities (following background removal/standardization). The resulting output is a measurement of overall staining intensity per cell for comparison between treatment groups.

3.5 *Statistical Analysis*

Statistical analysis was performed with Statistical Analysis Software (SAS Institute Inc., Cary, NC) and figures were made with Prism 6 (GraphPad Software, La Jolla, CA). Physiologic parameters were analyzed by their association with each observed timepoint and stratified by treatment group utilizing a Spearman Rank Correlation with results reported as (correlation coefficient, p-value). Shaprio-wilk test was used to evaluate data within each treatment group for distribution normality. Groups were compared using an unpaired T-test or ordinary one-way analysis of variance with multiple comparisons (ANOVA) (normally distributed) and Mann-Whitney test or Kruskal-Wallis with multiple comparisons (non-normally distributed) where relevant. A sample size of 6 per group was selected to detect a 20% difference in serum cytokine or biomarker concentration and histologic analysis with 10% standard deviation at 97.05% power assuming a two-way power analysis. Primary data is reported as mean (standard deviation).

4.0. RESULTS

4.1. IL-1β Cytokine Serum Analysis

Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-1β in the TBI-12,000-85 treatment group compared to Sham-Ground-100 (7.3 picogram/microliter (pg/uL) (3.0) vs. 3.9pg/uL (0.6), p=0.02), TBI-Ground-100 (7.3 pg/uL (3.0) vs. 2.5 pg/uL (0.79), p<0.01), and Sham-Ground-85 (7.3 pg/uL (3.0) vs. 3.8 pg/uL (1.6), p=0.01) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-1β in the TBI-12,000-100 treatment group when compared to Sham-Ground-100 (7.6 pg/uL (2.7) vs. 3.9 pg/uL (0.6), p<0.01), TBI-Ground-100 (7.6 pg/uL (2.7) vs. 2.5 pg/uL (0.79), p<0.01), and Sham-Ground-85 (7.6 pg/uL (2.7) vs. 3.8 pg/uL (1.6), p=0.01) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-1β in the Sham-Ground-100 treatment group compared to the TBI-Ground-100 treatment group (3.9 pg/uL (0.6) vs. 2.5 pg/uL (0.8), p<0.01). **(Figure 1A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-1β in the TBI-12,000-85 treatment group when compared to Sham-Ground-100 (10.2 pg/uL (8.9) vs. 3.7 pg/uL (0.7), p=0.03) and TBI-Ground-100 (10.2pg/uL (8.9) vs. 2.6pg/uL (1.0), p<0.01) treatment groups. Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-1β in the TBI-12,000-100 treatment group compared to Sham-Ground-100 (12.0 pg/uL (14.8) vs. 3.7 pg/uL (0.7), p=0.02), TBI-Ground-100 (12.0 pg/uL (14.8) vs. 2.6 pg/uL (1.0), p<0.01), and Sham-Ground-85

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(12.0 pg/uL (14.8) vs. 4.1 pg/uL (1.4), p=0.02) treatment groups. Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-1β in the Sham-Ground-100 treatment group compared to the TBI-Ground-100 treatment group (3.7 pg/uL (0.7) vs. 2.6 pg/uL (1.0), p=0.01). **(Figure 1B)**

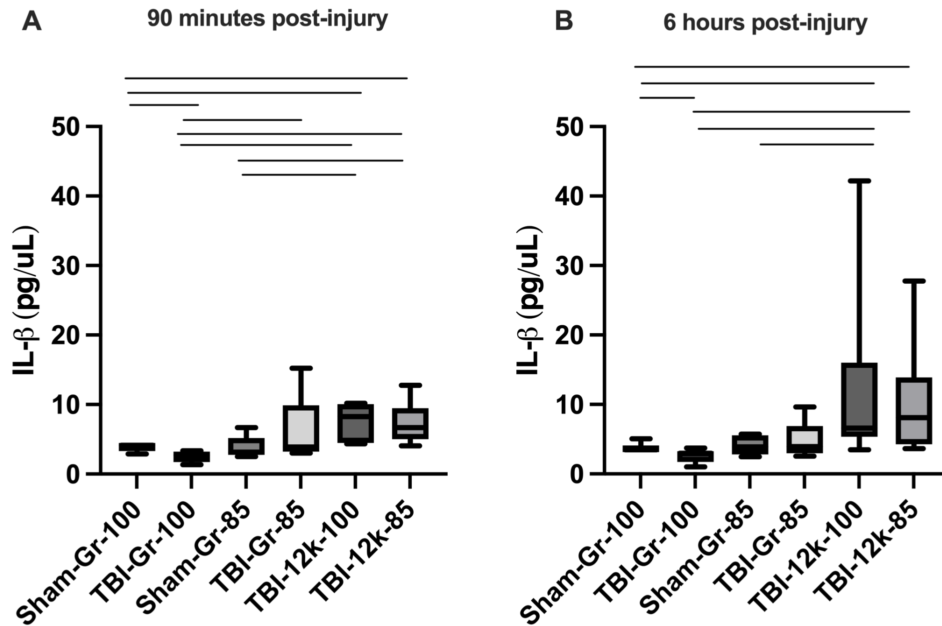

Figure 1. Serum levels of IL-1β
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-1β in the TBI-5k-95 (7.9 pg/uL (4.4) vs. 2.5 pg/uL (0.8), $p<0.01$) and TBI-5k-100 (28.7 pg/uL (41.4) vs. 2.5 pg/uL (0.8), $p=0.02$) treatment groups compared to the TBI-Ground-100 treatment group. **(Figure 2A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-1β in the TBI-5k-95 (10.9 pg/uL (2.9) vs. 2.6 pg/uL (1.0), $p<0.01$) and TBI-5k-100 (43.8 pg/uL (62.3) vs. 2.6 pg/uL (1.0), $p<0.01$) treatment groups compared to the TBI-Ground-100 treatment group. **(Figure 2B)**

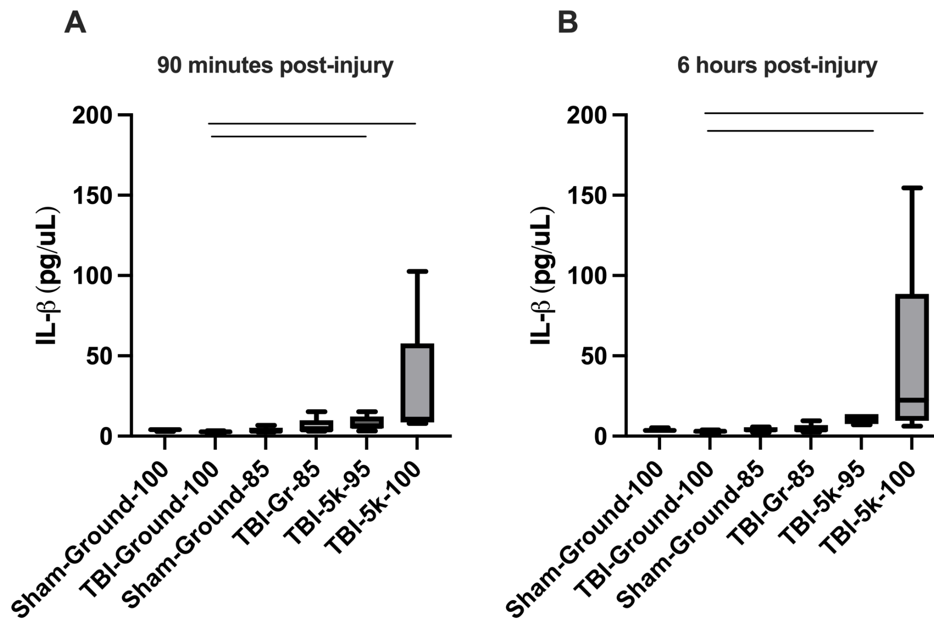

Figure 2. Serum levels of IL-1β
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-5,000 ft-95% SpO_2, TBI-5,000 ft-100% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-1β in the TBI-8k-90 (8.7 pg/uL (3.7) vs. 2.5 pg/uL (0.8), $p<0.01$) and TBI-8k-100 (16.0 pg/uL (9.9) vs. 2.5 pg/uL (0.8), $p<0.01$) treatment groups compared to the TBI-Ground-100 treatment group. **(Figure 3A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-1β in the TBI-8k-90 (17.1 pg/uL (13.0) vs. 2.6 pg/uL (1.0), $p<0.01$) and TBI-8k-100 (20.3 pg/uL (9.5) vs. 2.6 pg/uL (1.0), $p<0.01$) treatment groups compared to the TBI-Ground-100 treatment group. **(Figure 3B)**

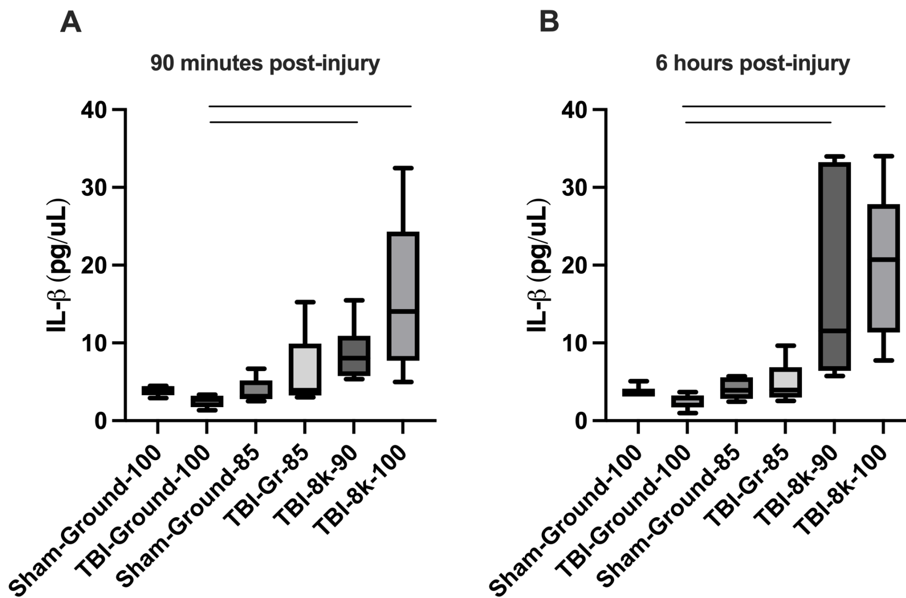

Figure 3. Serum levels of IL-1β
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-8,000 ft-90% SpO_2, TBI-8,000 ft-100% SpO_2.

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4.2 IL-6 Cytokine Serum Analysis

Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-12,000-85 treatment group compared to Sham-Ground-100 (122.9 pg/uL (93.9) vs. 9.0 pg/uL (2.8), p=0.01), TBI-Ground-100 (122.9 pg/uL (93.9) vs. 10.0 pg/uL (3.3), p=0.01), Sham-Ground-85 (122.9 pg/uL (93.9) vs. 9.8 pg/uL (3.9), p=0.01), TBI-Ground-85 (122.9 pg/uL (93.9) vs. 13.3 pg/uL (7.5), p=0.02) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-12,000-100 treatment group compared to Sham-Ground-100 (136.0 pg/uL (203.3) vs. 9.0 pg/uL (2.8), p<0.01), TBI-Ground-100 (136.0 pg/uL (203.3) vs. 10.0 pg/uL (3.3), p=0.02), and Sham-Ground-85 (136.0 pg/uL (203.3) vs. 9.8 pg/uL (3.9), p=0.02) treatment groups. **(Figure 4A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-6 in the TBI-12,000-85 treatment group compared to Sham-Ground-100 (20.9 pg/uL (9.0) vs. 10.8 pg/uL (3.8), p=0.03), TBI-Ground-100 (20.9 pg/uL (9.0) vs. 11.2 pg/uL (1.6), p=0.03), Sham-Ground-85 (20.9 pg/uL (9.0) vs. 10.4 pg/uL (3.2), p=0.02), and TBI-Ground-85 (20.9 pg/uL (9.0) vs. 10.6 pg/uL (3.1), p=0.02) treatment groups. Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-6 in the TBI-12,000-100 treatment group compared to Sham-Ground-100 (84.6 pg/uL (125.3) vs. 10.8 pg/uL (3.8), p<0.01), TBI-Ground-100 (84.6 pg/uL (125.3) vs.11.2 pg/uL (1.6), p<0.01), Sham-Ground-85 (84.6 pg/uL (125.3) vs. 10.4 pg/uL (3.2), p<0.01), and TBI-Ground-85 (84.6 pg/uL (125.3) vs. 10.6 pg/uL (3.1), p<0.01) treatment groups. **(Figure 4B)**

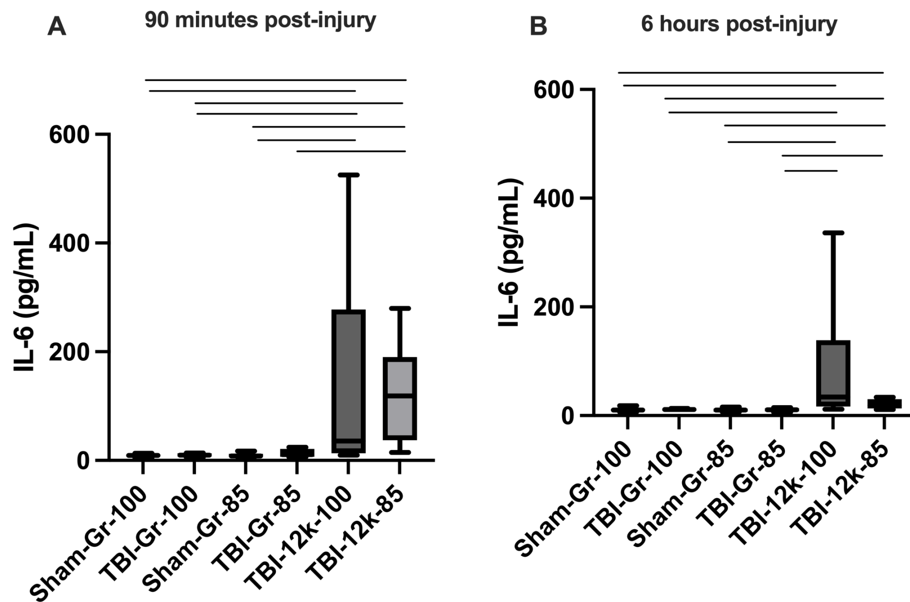

Figure 4. Serum levels of IL-6

(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-5k-95 treatment group compared to the Sham-Ground-100 (54.9 pg/uL (30.8) vs. 9.0 pg/uL (2.8), p=0.04) and Sham-Ground-85 (54.9 pg/uL (30.8) vs. 9.8 pg/uL (3.9), p=0.04) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-5k-100 treatment group compared to the Sham-Ground-100 (106.6 pg/uL (123.9) vs. 9.0 pg/uL (2.8), p=0.02), TBI-Ground-100 (106.6 pg/uL (123.9) vs. 9.9 pg/uL (3.3), p=0.04), and Sham-Ground-85 (106.6 pg/uL (123.9) vs. 9.8 pg/uL (3.9), p=0.03) treatment groups. **(Figure 5A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-6 in the TBI-5k-95 treatment group compared to the Sham-Ground-100 (39.2 pg/uL (9.5) vs. 10.8 pg/uL (3.8), p<0.01), TBI-Ground-100 (39.2 pg/uL (9.5) vs. 11.2 pg/uL (1.6), p<0.01), Sham-Ground-85 (39.2 pg/uL (9.5) vs. 10.4 pg/uL (3.2), p<0.01), and TBI-Ground-85 (39.2 pg/uL (9.5) vs. 10.6 pg/uL (3.1), p<0.01) treatment groups. Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-6 in the TBI-5k-100 treatment group when compared to the Sham-Ground-100 (41.8 pg/uL (11.3) vs. 10.8 pg/uL (3.8), p<0.01), TBI-Ground-100 (41.8 pg/uL (11.3) vs. 11.2 pg/uL (1.6), p<0.01), Sham-Ground-85 (41.8 pg/uL (11.3) vs. 10.4 pg/uL (3.2), p<0.01), and TBI-Ground-85 (41.8 pg/uL (11.3) vs. 10.6 pg/uL (3.1), p<0.01) treatment groups. **(Figure 5B)**

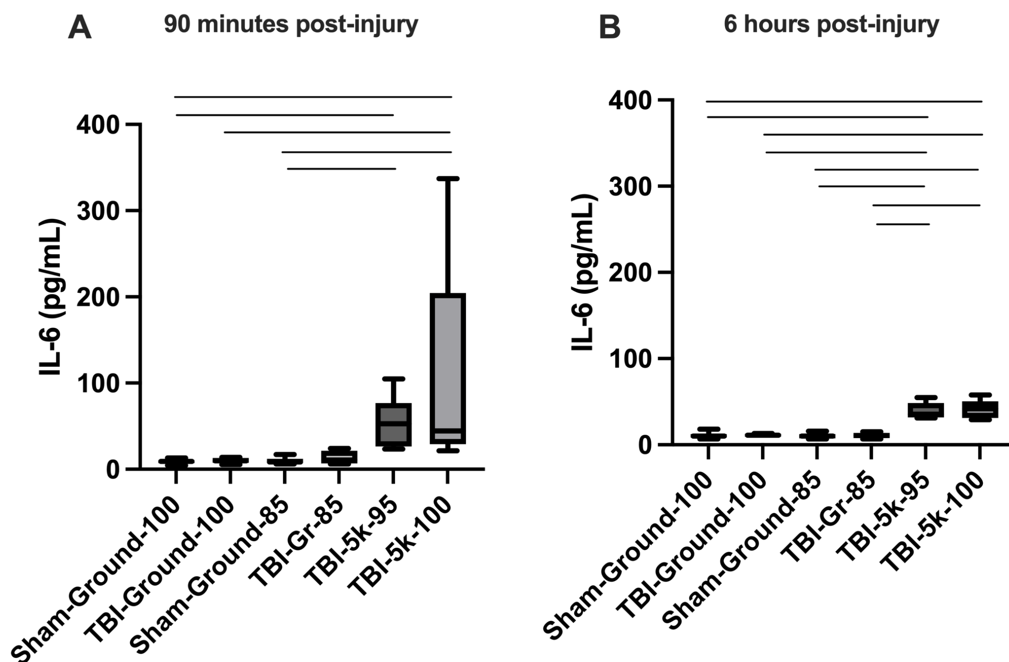

Figure 5. Serum levels of IL-6
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-5,000 ft-95% SpO_2, TBI-5,000 ft-100% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-8k-90 treatment group compared to the Sham-Ground-100 (124.2 pg/uL (137.7) vs. 9.0 pg/uL (2.8), p=0.02), TBI-Ground-100 (124.2 pg/uL (137.7) vs. 9.9 pg/uL (3.3), p=0.03), and Sham-Ground-85 (124.2 pg/uL (137.7) vs. 9.8 pg/uL (3.9), p=0.02) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of IL-6 in the TBI-8k-100 treatment group compared to the Sham-Ground-100 (68.0 pg/uL (28.6) vs. 9.0 pg/uL (2.8), p=0.04) and Sham-Ground-85 (68.0 pg/uL (28.6) vs. 9.8 pg/uL (3.9), p=0.04) treatment groups. **(Figure 6A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of IL-6 in the TBI-8k-90 treatment group compared to the Sham-Ground-100 (64.7 pg/uL (44.5) vs. 10.8 pg/uL (3.8), p<0.01), TBI-Ground-100 (64.7 pg/uL (44.5) vs. 11.2 pg/uL (1.6), p<0.01), Sham-Ground-85 (64.7 pg/uL (44.5) vs. 10.4 pg/uL (3.2), p<0.01), and TBI-Ground-85 (64.7 pg/uL (44.5) vs. 10.6 pg/uL (3.1), p<0.01) treatment groups. **(Figure 6B)**

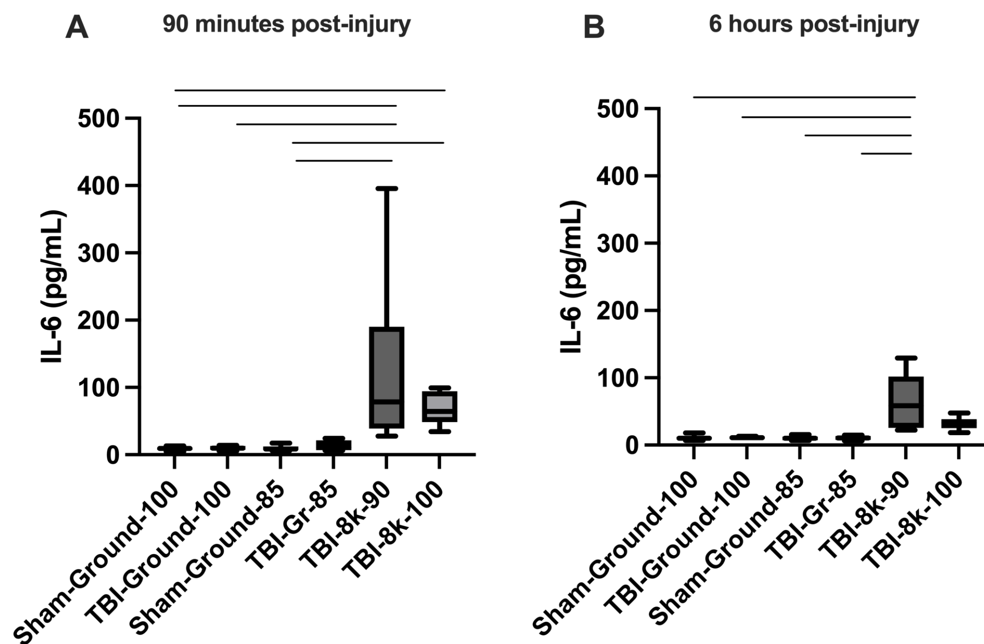

Figure 6. Serum levels of IL-6
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-8,000 ft-90% SpO_2, TBI-8,000 ft-100% SpO_2.

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4.3 TNF-α Cytokine Serum Analysis
Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-12,000-85 treatment group compared to Sham-Ground-100 (444.4 pg/uL (390.3) vs. 29.4 pg/uL (17.3), p=0.03), TBI-Ground-100 (444.4 pg/uL (390.3) vs. 22.9 pg/uL (7.8), p=0.02), and Sham-Ground-85 (444.4 pg/uL (390.3) vs. 25.2 pg/uL (12.1), p=0.03) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-12,000-100 treatment group compared to Sham-Ground-100 (506.2 pg/uL (435) vs. 29.4 pg/uL (17.3), p<0.01), TBI-Ground-100 (506.2 pg/uL (435) vs. 22.9 pg/uL (7.8), p<0.01), and Sham-Ground-85 (506.2 pg/uL (435) vs. 25.2 pg/uL (12.1), p<0.01) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-Ground-85 treatment group compared to TBI-Ground-100 (191.9 pg/uL (347.6) vs. 22.9 pg/uL (7.8), p=0.03) and Sham-Ground-85 (191.9 pg/uL (347.6) vs. 25.2 pg/uL (12.1), p=0.04) treatment groups. **(Figure 7A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of TNF-α in the TBI-12,000-100 treatment group compared to TBI-Ground 100 (61.2 pg/uL (38.3) vs. 17.4 pg/uL (11.1), p=0.02) and Sham-Ground-85 (61.2 pg/uL (38.3) vs. 19.9 pg/uL (9.8), p=0.03) treatment groups. Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of TNF-α in the TBI-Ground-85 treatment group compared to TBI-Ground-100 (55.4 pg/uL (48.6) vs. 17.4 pg/uL (11.1), p=0.03) and Sham-Ground-85 (55.4 pg/uL (48.6) vs. 19.9 pg/uL (9.8), p=0.04) treatment groups. **(Figure 7B)**

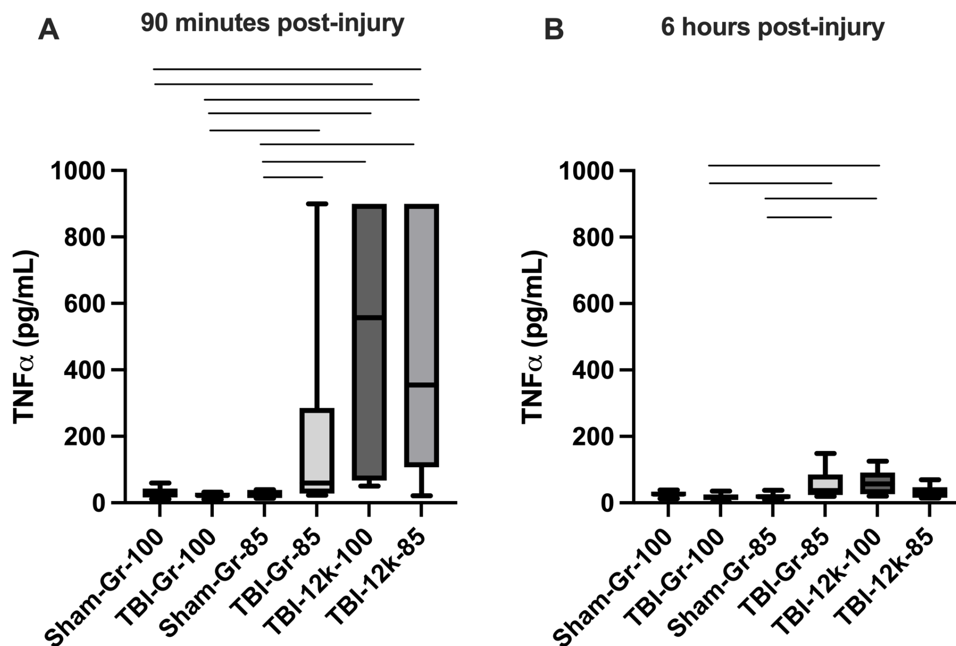

Figure 7. Serum levels of TNF-α
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-5k-95 treatment group compared to the TBI-Ground-100 treatment group (691.2 pg/uL (1031.0) vs. 22.9 (7.8), p=0.05). Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-5k-100 treatment group compared to the Sham-Ground-100 (1539.2 pg/uL (1292.2) vs. 29.4 pg/uL (17.3), p=0.02), TBI-Ground-100 (1539.2 pg/uL (1292.2) vs. 22.9 pg/uL (7.8), p<0.01), and Sham-Ground-85 (1539.2 pg/uL (1292.2) vs. 25.2 pg/uL (12.1), p=0.01) treatment groups. **(Figure 8A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated no significant difference in TNF-α concentrations when considering groups exposed to 5,000 ft of altitude. **(Figure 8B)**

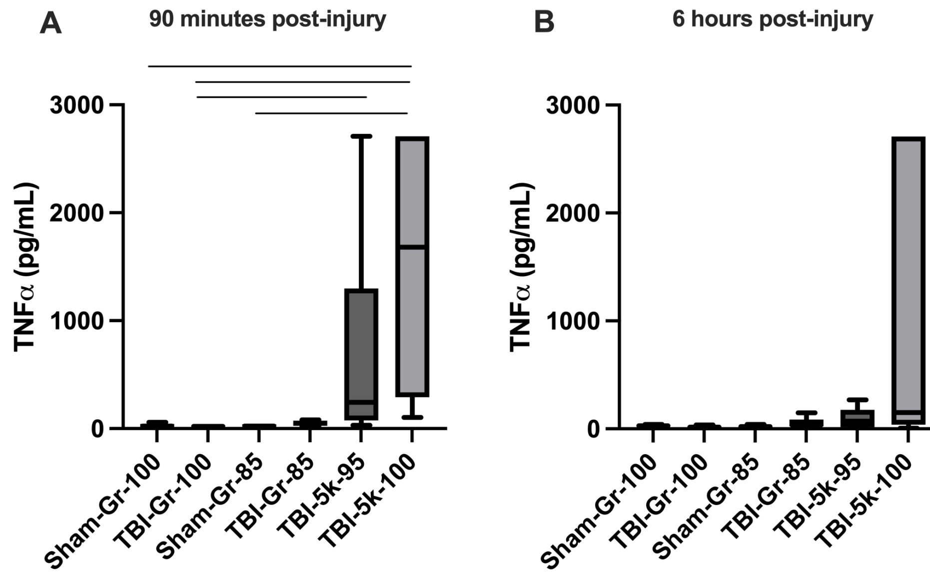

Figure 8. Serum levels of TNF-α

(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-5,000 ft-95% SpO_2, TBI-5,000 ft-100% SpO_2.

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Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-8k-90 treatment group compared to the TBI-Ground-100 (1420.6 pg/uL (1410.7) vs. 22.9 pg/uL (7.8), p=0.02) and Sham-Ground-85 (1420.6 pg/uL (1410.7) vs. 25.2 pg/uL (12.1), p=0.03) treatment groups. Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of TNF-α in the TBI-8k-100 treatment group compared to the Sham-Ground-100 (2015.1 pg/uL (1079.0) vs. 29.4 pg/uL (17.3), p=0.02), TBI-Ground-100 (2015.1 pg/uL (1079.0) vs. 22.9 pg/uL (7.8), p<0.01), and Sham-Ground-85 (2015.1 pg/uL (1079.0) vs. 25.2 pg/uL (12.1), p<0.01) treatment groups. **(Figure 9A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated significantly higher levels of TNF-α in the TBI-8k-100 treatment group compared to the TBI-Ground-100 (153.3 pg/uL (110.4) vs. 17.4 pg/uL (11.1), p=0.02) and Sham-Ground-85 (153.3 pg/uL (110.4) vs. 19.9 pg/uL (9.8), p=0.04) treatment groups. **(Figure 9B)**

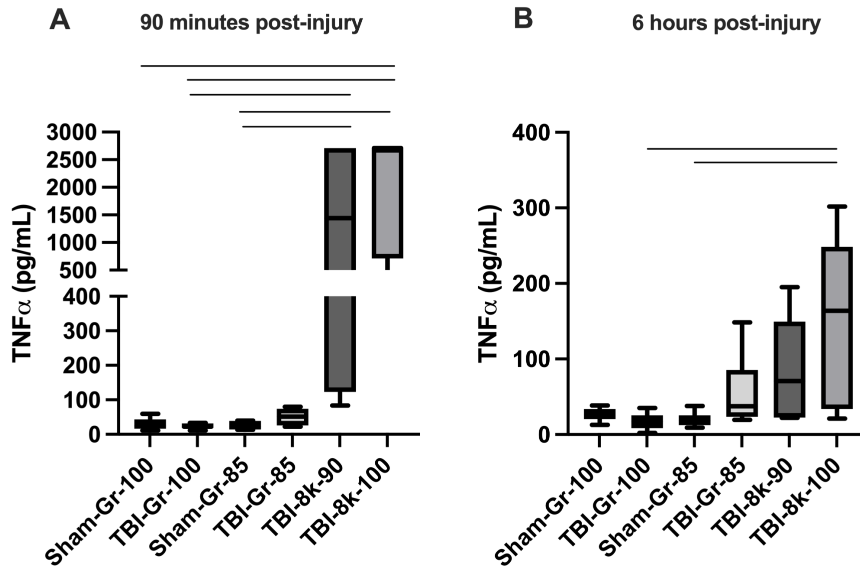

Figure 9. Serum levels of IL-6
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-8,000 ft-90% SpO_2, TBI-8,000 ft-100% SpO_2.

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4.4 IL-8 Cytokine Serum Analysis
Serum collected immediately post-flight and at 6 hours post-injury demonstrated no significant differences in IL-8 between treatment groups.

4.5 HIF-1α Serum Analysis
Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of HIF1α in the TBI-12,000-85 treatment group compared to Sham-Ground-100 (485.2 pg/uL (166.9) vs. 257.2 pg/uL (38.2), p=0.02), TBI-Ground-100 (485.2 pg/uL (166.9) vs. 257.3 pg/uL (52.1), p<0.01), Sham-Ground-85 (485.2pg/uL (166.9) vs. 306.5 pg/uL (75.2), p=0.04), TBI-Ground-85 (485.2 pg/uL (166.9) vs. 274.5 pg/uL (52.0), p=0.01), TBI-12,000-100 (485.2 pg/uL (166.9) vs. 308.7 pg/uL (56.1), p=0.03) treatment groups. **(Figure 10A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated no significant differences in HIF1α between treatment groups. **(Figure 10B)**

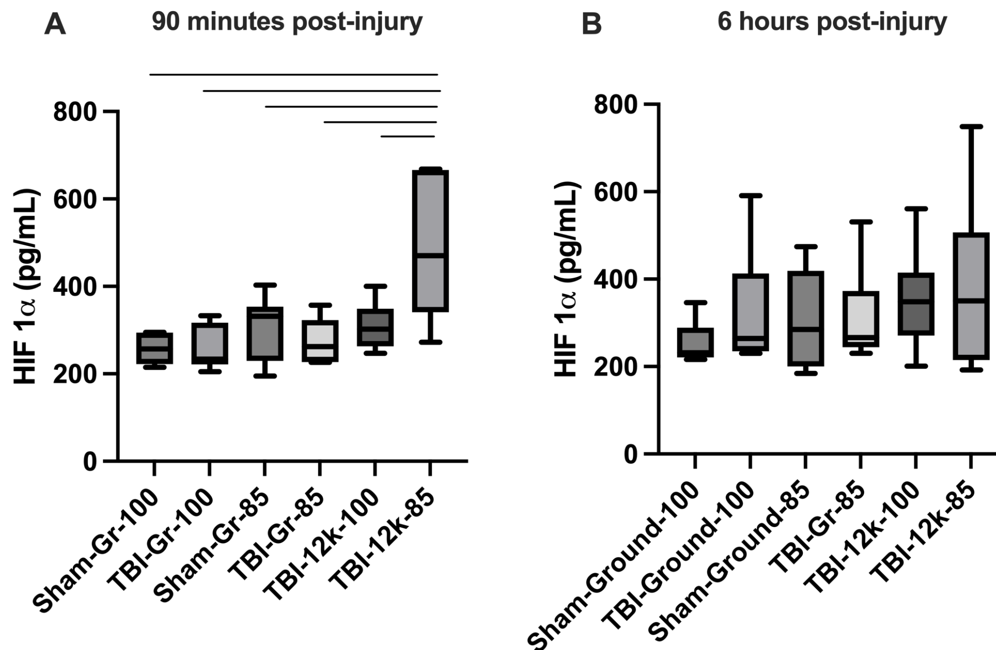

Figure 10. Serum levels of HIF1-α
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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4.6 UCH-L1 Serum Analysis

Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of UCH-L1 in the Sham-Ground-100 treatment group compared to the TBI-12,000-85 treatment group (2.0 ng/uL (2.3) vs. 1.7ng/uL (0.2), p=0.04). **(Figure 11A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated no significant differences in UCH-L1 between treatment groups. **(Figure 11B)**

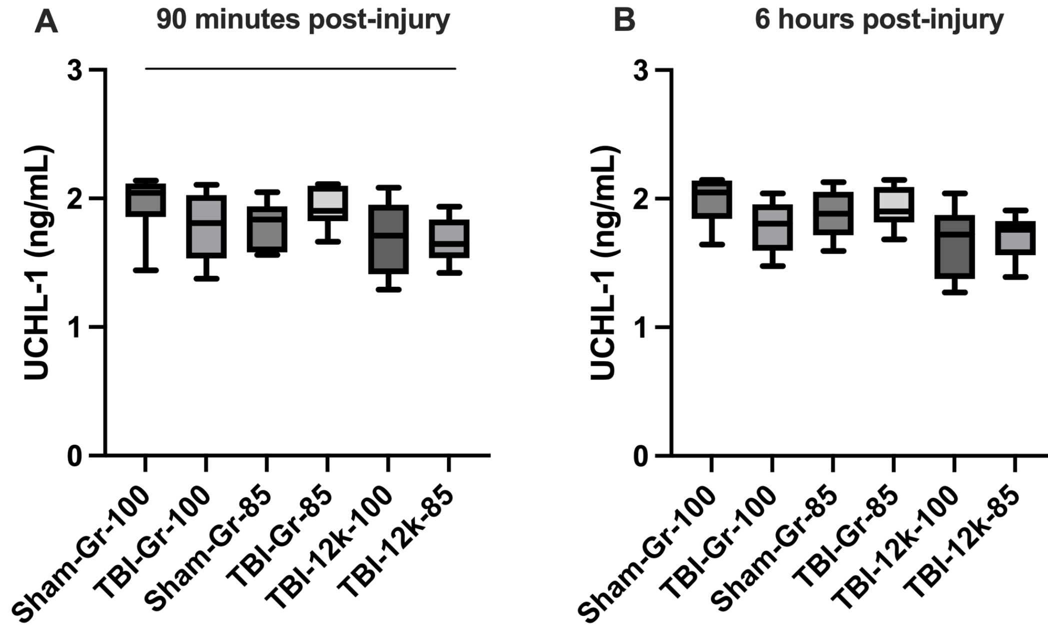

Figure 11. Serum levels of UCH-L1
(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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4.7 NSE Serum Analysis

Serum collected immediately post-flight (approximately 90 minutes post-injury) demonstrated significantly higher levels of NSE in the TBI-Ground-85 treatment group compared to TBI-Ground 100 (9. 7ng/uL (2.7) vs. 6.7 ng/uL (1.8), p=0.05) and TBI-12,000-85 (9.7ng/uL (2.7) vs. 6.8ng/uL (2.3), p=0.02) treatment groups. **(Figure 12A)** Serum collected at 6 hours post-injury (4.5 hours post-flight) demonstrated no significant differences in NSE between treatment groups. **(Figure 12B)**

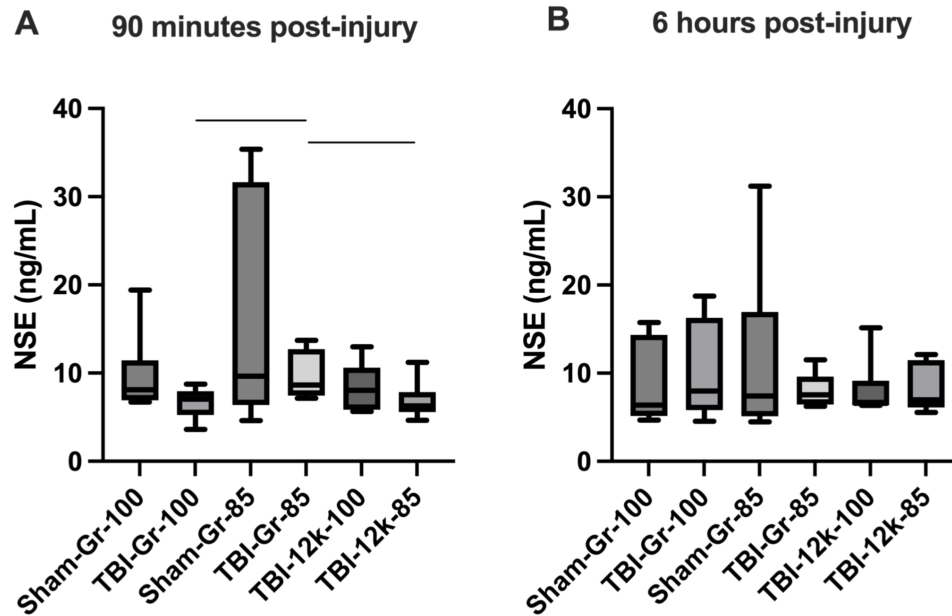

Figure 12. Serum levels of NSE

(A) 90-minute post-injury timepoint and (B) 6-hour post-injury timepoint for treatment groups Sham-Ground-100% SpO_2, TBI-Ground-100% SpO_2, Sham-Ground-85% SpO_2, TBI-Ground-85% SpO_2, TBI-12,000 ft-100% SpO_2, and TBI-12,000 ft-85% SpO_2.

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4.8 Amyloid A Serum Analysis
Serum collected immediately post-flight (approximately 90 minutes post-injury) and at 6 hours post-injury (4.5 hours post-flight) demonstrated no significant differences in Amyloid A concentration between treatment groups.

4.9 Histologic Analysis
Histologic examination involving the staining intensity ratio of injured to uninjured cortex p-tau did not differ significantly between the two most extreme treatment groups, Sham-Ground-100 and TBI-12,000-85 (p=0.33) **(Figure 13A)** Histologic examination involving the staining intensity ratio of injured to uninjured hippocampus p-tau did not differ significantly between the two most extreme treatment groups, Sham-Ground-100 and TBI-12,000-85 (p=0.29) **(Figure 13B)**

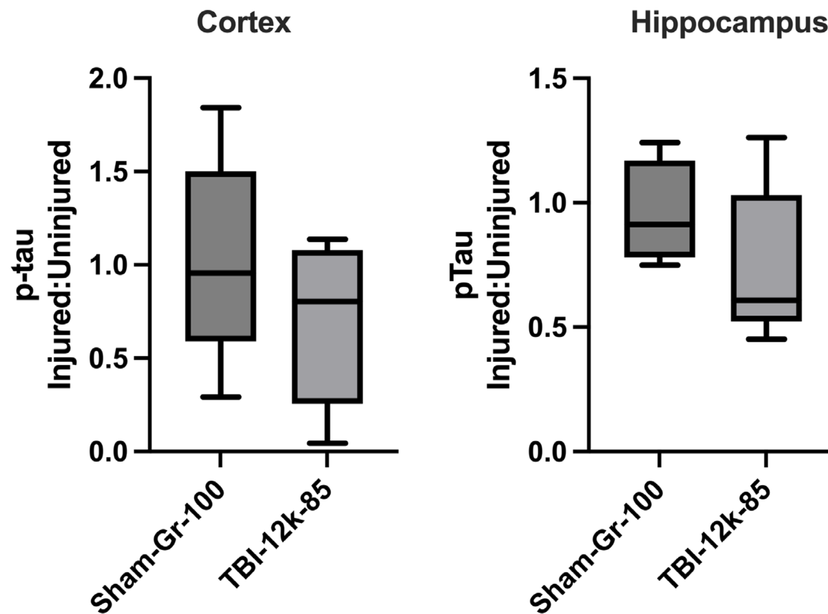

Figure 13. Histologic analysis of brain tissue demonstrated no significant difference in the ratio of p-tau protein staining intensity between injured and uninjured sides of the brain between Sham-Ground-100 and TBI-12,000-85 Groups in either cortex (A) or hippocampal (B) samples.

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Histologic examination involving the staining intensity ratio of injured to uninjured cortex GFAP did not differ significantly between the two most extreme treatment groups, Sham-Ground-100 and TBI-12,000-85 (p=0.92) **(Figure 14A)** Histologic examination involving the staining intensity ratio of injured to uninjured hippocampus GFAP did not differ significantly between the two most extreme treatment groups, Sham-Ground-100 and TBI-12,000-85 (p=0.10) **(Figure 14B)**

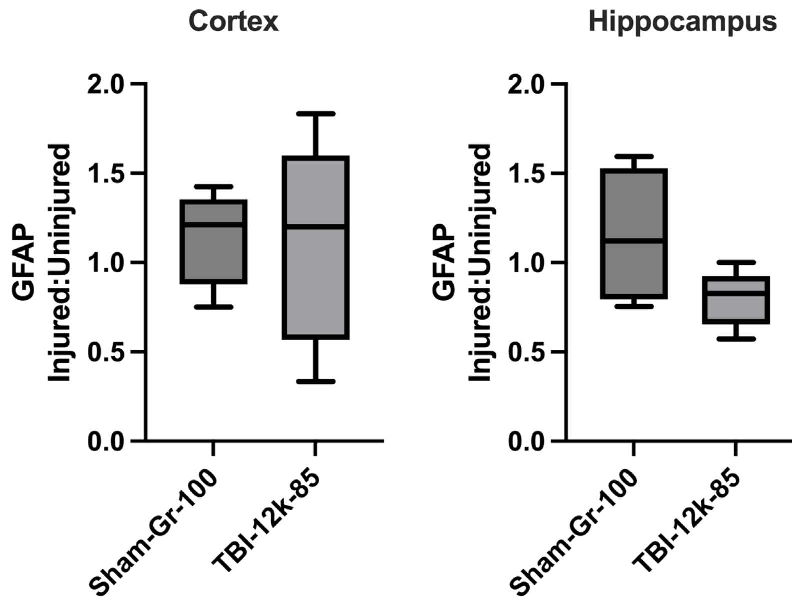

Figure 14. Histologic analysis of brain tissue demonstrated no significant difference in the ratio of GFAP protein staining intensity between injured and uninjured sides of the brain between Sham-Ground-100 and TBI-12,000-85 Groups in either cortex (A) or hippocampal (B) samples.

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4.10 Brain parenchymal blood flow and tissue oxygenation
Analysis of brain parenchymal blood flow as a ratio of injured to uninjured brain parenchyma did not differ significantly throughout the course of the study in either the Sham-Griund-100 or TBI-12,000-85 treatment groups. **(Figure 15A)** Analysis of brain parenchymal tissue oxygenation as a ratio of injured to uninjured brain parenchyma did not differ significantly throughout the course of the study in either the Sham-Griund-100 or TBI-12,000-85 treatment groups. **(Figure 15B)**

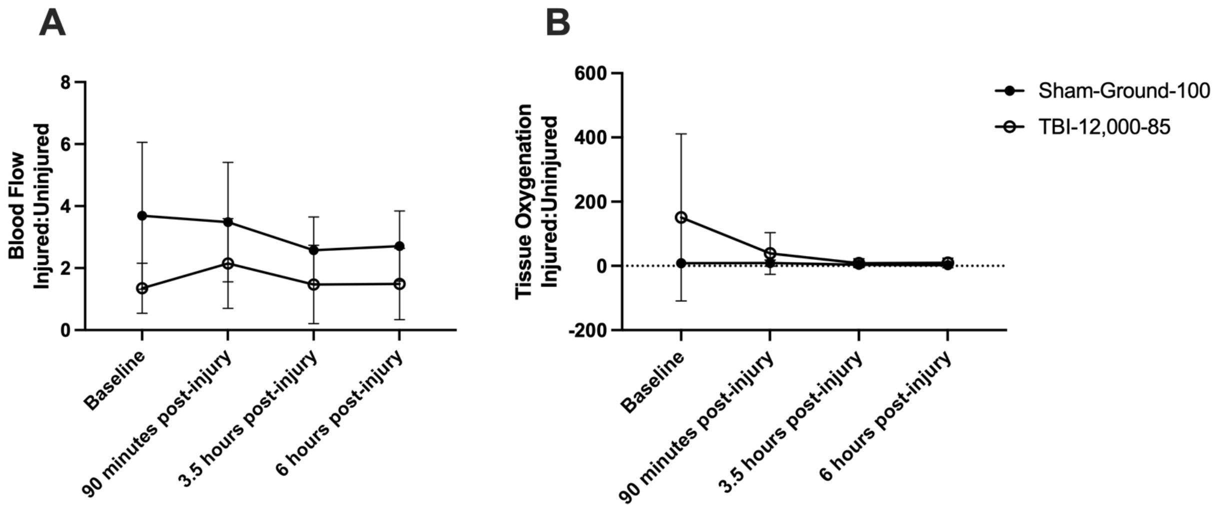

Figure 15. Analysis of brain parenchymal
(A) blood flow as measured with Oxyflo probes and (B) tissue oxygenation by way of OxyLite probes at the timepoints of baseline, 90 minutes post-injury, 3.5 hours post-injury, and 6 hours post-injury between groups Sham-Ground-100% SpO_2 and TBI-12,000ft-85% SpO_2.

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5.0. DISCUSSION

In the present study, we investigated the effect of AE conditions of hypobaria and hypoxia on serum inflammatory cytokine release and markers of brain injury severity following TBI. We determined that exposure to the hypobaric environment of AE increased inflammatory cytokines IL-1β, IL-6, and TNFα at timepoints of 90 minutes and 6 hours post-injury, in both hypoxic and normoxic conditions when compared to TBI under normoxic conditions with no hypobaric exposure. This elevation in systemic cytokines was present at altitudes of 12,000ft, 8,000ft, and 5,000ft. Further, we demonstrated a significant elevation in HIF1α following TBI with combined hypobaric and hypoxic exposure compared to all other treatment groups, including those undergoing TBI with hypobaric exposure under normoxic conditions. Finally, we demonstrated that despite an increase in systemic inflammation with hypobaric exposure, no changes in tissue oxygenation or blood flow was observed within the brain parenchyma following TBI with hypobaric and hypoxic exposure compared to the treatment group following sham TBI and no hypobaric exposure under normoxic conditions. Importantly, none of the changes induced by 12,000ft, 8,000ft, or 5,000ft. of altitude exposure were mitigated by the normalization of systemic oxygenation. Additionally, in this non-survival study, hypoxia alone did not independently contribute to the physiologic or inflammatory injury following TBI.

Modern AE involves the transport of critically ill patients from far-forward combat or austere civilian environments to centers of definitive care, generally dependent on rotary-wing (helicopter) aircraft, fixed-wing (airplane) aircraft, or a combination of the two. While AE via rotary-wing aircraft allows for a higher degree of maneuverability and flexibility in landing locations, they are inherently limited in regard to maximum altitude, speed, and distance of transport. AE via fixed-wing aircraft, however, offers the ability to travel longer distances in a shorter period of time toward higher levels of definitive care for critically injured patients.[6–8] It is important to the present study to consider the difference in maximum altitude, or the true distance above sea-level, and cabin altitude, or physiologic level of hypobaria accounting for cabin pressurization, between these two types of aircraft. Given the inherent inability for most rotary-wing aircraft to pressurize the cabin, the maximum altitude and cabin altitude are equivalent. Despite rare indications for these rotary-wing aircraft to surpass an altitude of 10,000ft, military helicopter crews are required to undergo regular altitude hypoxia training at 18,000ft.[14] Fixed-wing aircraft achieve maximum altitudes in excess of 30,000ft with cabin altitudes generally limited to 8,000 ft via cabin pressurization.[12] The degree of hypoxemia at the cabin altitude of 8,000ft may be under-appreciated, with prior research by Cottrell, et al. demonstrating that cabin pressurization of commercial flights to 5,000-8,000ft results in oxygen saturation of 89% in healthy crew members.[15] For the purposes of the present study, we selected an altitude of 12,000ft as an intentionally extreme but plausible altitude for realistic AE to investigate the effect of hypobaria and associated hypoxia following TBI as may occur in depressurized cabins or drone-related AE of the near future.[16]

TBI is associated with a well-studied systemic inflammatory cascade aimed at neuroprotection, repair, and pathogen defense that may act paradoxically in the setting of blood-brain barrier (BBB) disruption from primary injury.[17,18] Following head injury, cytokines are secreted both systemically and from within the central nervous system (CNS), representing both harm via inflammatory propagation and exacerbation of hypoxemia, and protection via neurologic regenerative properties.[19] Hypoxemia is among the most well-established sources of secondary injury that lead to worse neurocognitive outcomes in patients with moderate to severe TBI.[20–22] Given that hypoxemia is an independent source of systemic inflammation in the absence of TBI, there may be a synergistic relationship between hypoxemia and systemic inflammation in worsening secondary injury in the acute TBI patient.[9,10] Although the mechanism by which altitude causes an increase in systemic inflammation remains unclear, there is a hypothesized connection to hypobaria-associated hypoxia and subsequent passenger hypoxemia.[12] In the present study, we demonstrated that exposure to altitude as high as 12,000ft. and as low as 5,000ft. during AE

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immediately following TBI increases inflammatory cytokines IL-1β, IL-6, and TNFα for up to six hours in both hypoxic and normoxic conditions. This observation alone indicates that the systemic inflammation following TBI is exacerbated by the hypobaric conditions of flight independent of passenger hypoxemia. Subsequently, the environment of AE has the potential to worsen secondary injury after TBI even with the appropriate avoidance of hypoxemia in the acute post-traumatic setting. Our observations regarding the level of altitude exposure is integral to our analysis. Given that the federally-mandated cabin pressure ceiling is 8,000ft. for commercial flight and those critically-injured in the military may be prescribed Cabin Altitude Restriction (altitude exposure limited to 4,000ft.-6,000ft.), the effect of systemic inflammation at 5,000ft. and 8,000ft. of altitude were also evaluated.[23] A similar effect of increased inflammation with hypobaric exposure alone (with or without concomitant hypoxia) existed within these altitude-limited treatment groups. This rise in inflammatory cytokine release by the measure of IL-1β, IL-6, and TNFα was present immediately following altitude exposure and in several cases persisted throughout the 4.5-hour observation period. The acute timing of our observed increase in systemic inflammatory response following TBI is also notable, given that delayed AE following TBI may be a reasonable strategy to avoiding this source of secondary injury. Maddry et al. previously demonstrated better long-term outcomes in TBI patients who underwent AE on the second or third day following TBI when compared to those who underwent immediate air transport.[24]

It is also important to consider the specific roles that the observed inflammatory mediators in this study, IL-1β, IL-6, and TNFα play in worsening of secondary injury following TBI. IL-1β, the secreted variant of IL-1, plays a role in microglial, astrocyte, and neuronal signaling via upregulated receptors following acute brain injury. There is a specific mechanistic relationship between IL-1 and the breakdown of the BBB via metalloprotease upregulation, contributing to pathologic exposure of brain tissue to systemic circulatory inflammation following injury.[12,19] IL-6 interestingly demonstrates both neuroprotective and damaging properties in the setting of TBI. When maintained within the CNS, IL-6 demonstrated anti-inflammatory effect via regulation of TNFα. However, breakdown of the BBB and systemic release of IL-6 leads to release of other inflammatory mediators and hepatic reactants that then have the potential to worsen neuroinflammation.[12,17,19] Finally, TNFα is known to increase inflammatory cellular mediators to the CNS, catalyze BBB breakdown, and inhibit neuronal regeneration.[12,25,26] Taken together, patients with increased CNS levels of IL-1β, serum levels of IL-6, and serum levels of TNFα post-injury demonstrate worse neurocognitive recovery.[12,17,19,25,26]

HIF-1α is another dual-effect contributor to TBI by either neuroprotection or worsened secondary injury. While HIF-1α stabilization has been shown to contribute to improved neurocognitive outcomes, data from Fang et al. have shown that HIF-1α is implicated in inflammation-related neuronal apoptosis.[27,28] Further data has supported the implication of HIF-1α in neuroinflammation and neuron death following TBI as a potential catalyst for more severe secondary injury.[29] Our data demonstrate an increase in HIF-1α in the treatment group exposed to both hypobaria and hypoxia following TBI compared to all other treatment groups. This finding was notably and uniquely present only at the 90-minute post-injury (immediate post-flight) timepoint and not at the 6-hour post-injury timepoint. The timing of the increase in HIF-1α and subsequent return to levels commensurate with control conditions suggests that a systemic increase in HIF-1α is an acute and transient effect that may only be present during the time of flight and for a short period after aircraft landing. Given the known mechanisms of injury related to HIF-1α in TBI, this may represent a mechanism for worsened secondary injury that is independent of the known inflammatory cytokine release and therefore an additional target for intervention.

Finally, our analysis included the measurement of several potential biomarkers previously demonstrated to have clinical relevance in TBI, including UCH-L1, NSE, and Amyloid A. Serum UCH-L1, NSE, and Amyloid A have all previously been demonstrated as effective prognostic indicators in patients with

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TBI.[30–34] In the present study, these biomarkers did not demonstrate elevation in response to hypobaric or hypoxic conditions. Notably, they did not demonstrate a statistically or clinically meaningful difference under normal (normoxic, ground level) conditions with TBI when compared to sham TBI. We believe that the lack of efficacy of these biomarkers in identifying TBI in this study represents an ongoing challenge to find appropriate biomarkers for TBI severity in a porcine model, specifically and represents a target for future work.

This study does have limitations to be addressed. First, the nature of the CCI model of TBI utilized in the study may not be representative of all blunt trauma TBI and may influence the release systemic cytokines. This model was chosen, however, given its reproducibility across all animal models and our ability to eliminate confounding via TBI severity variation between pigs. Second, we were limited by porcine anatomy in that SpO_2 probes intended for use on human digits were placed on the tongue. Prior literature does implicate the location of probe placement in the variability of SpO_2 reading.[35] Finally, despite our attempts to analyze biomarkers shown in human patients to correlate with TBI severity in the context of porcine TBI, such biomarkers were not efficacious in detecting injury severity under control conditions in our porcine model. Future studies will be aimed at isolating an appropriate porcine TBI severity biomarker to correlate the observed systemic inflammatory response to TBI severity.

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6.0. CONCLUSION

In conclusion, our study demonstrates that the hypobaric environment of AE worsens the systemic release of several neuroinflammatory cytokines and may contribute to secondary injury following TBI. This systemic inflammatory response to AE is not improved by the mitigation of hypoxemia via supplemental oxygen or via the strategy of Cabin Altitude Restriction. Further studies are warranted to analyze the systemic inflammatory response following TBI and delayed hypobaric exposure, representing delayed AE following traumatic injury.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

Symbol	Definition
%	percent
<	less than
=	equals
>	greater than
×2	twice
°C	degrees Celsius
cm	centimeter
AE	aeromedical evacuation
BBB	blood-brain barrier
CCI	controlled cortical injury
CNS	central nervous system
DAB	Diaminobenzidine
ELISA	enzyme linked immunosorbent assay
FiO_2	Fraction of inspired oxygen
ft	feet
g	gravitational-force
GFAP	glial fibrillary acidic protein
HIF-1α	Hypoxia Inducible Factor 1α
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-6	interleukin-6
IL-8	interleukin 8
IL-8	interleukin
mm	millimeter
NSE	Neuron-specific Enolase
p	probability
p-tau	phosphorylated tau proteins
pg/uL	picogram/microliter
SAS	statistical analysis system
SpO2	oxygen saturation
TBI	traumatic brain injury
TBS-T	tris buffered saline with tween
TNF-α	tumor necrosis factor-alpha
TNF-α	tumor necrosis factor alpha
UCH-L1	Ubiquitin C-terminal Hydrolase L1

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