**Title:** Simulated Aeromedical Evacuation Exacerbates Experimental Brain Injury

**Running Title:** Hypobaria Exacerbates Experimental Brain Injury

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Abstract

Aeromedical evacuation, an important component in the care of many traumatic brain injury patients, particularly in war zones, exposes them to prolonged periods of hypobaria. The effects of such exposure on pathophysiological changes and outcome following traumatic brain injury are largely unexplored. The objective of this study was to investigate whether prolonged hypobaria in rats subjected to traumatic brain injury alters behavioral and histological outcomes. Adult male Sprague-Dawley rats were subjected to fluid percussion induced injury at 1.5-1.9 atmospheres of pressure. The effects of hypobaric exposure (6 hour duration; equivalent to 0.75 atmospheres) at 6, 24, and 72 hours, or 7 days after TBI were evaluated with regard to sensorimotor, cognitive and histological changes. Additional groups were evaluated to determine the effects of two hypobaric exposures after traumatic brain injury, representing primary simulated aeromedical evacuation (6 hour duration at 24 hours after injury) and secondary evacuation (10 hour duration at 72 hours after injury), as well as the effects of 100% inspired oxygen concentrations during simulated evacuation. Hypobaric exposure up to 7 days following injury significantly worsened cognitive deficits, hippocampal neuronal loss and microglial/astrocyte activation in comparison to injured controls not exposed to hypobaria. Hyperoxia during hypobaric exposure, or two exposures to prolonged hypobaric conditions further exacerbated spatial memory deficits. These findings indicate that exposure to prolonged hypobaria up to 7 days after traumatic brain injury, even while maintaining physiological oxygen concentration, worsens long-term cognitive function and neuroinflammation. Multiple exposures or use of 100% oxygen further exacerbates these pathophysiological effects.
Keywords

Traumatic brain injury, Inflammation, Neuronal cell death, Aeromedical Evacuation, Hypobaria.
Introduction

Traumatic Brain Injury (TBI) is a major public health issue, as well as an important concern for the military services. TBI is one of the leading causes of death and long-term disability associated with recent military conflicts. Isolated TBI represented 8% of all war-related wounds during Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). During these conflicts injuries to the head and neck ranked as the second most frequently injured body regions, occurring in 52% of all battle injuries and 22% of all non battle injury patients. Approximately 25% of all injured soldiers evacuated during OIF/OEF suffered head or neck injuries; this percentage is higher than for other major military operations of the 20th century and has lead to TBI being labeled the signature injury of these conflicts.  

The safe and effective movement of TBI patients from the battlefield to the appropriate level of care is critical for increasing survival and improving long term neurological outcomes. The US military Joint Theatre Trauma System (JTTS) ensures that combat casualties are transported as quickly as possible through several levels of resuscitative and surgical care, with the principal goal of preventing the development of secondary injury; for TBI patients emphasis is on control of intracranial pressure (ICP) and avoidance of hypotension or hypoxemia. An important component of the JTTS is the aeromedical evacuation system (AE). Although current AE practices have been successful at increasing survival from different types of battle wounds, concerns have been raised that for TBI patients AE itself may pose an added risk. The physiological stressors associated with flight may serve to exacerbate secondary injury mechanisms, adversely influencing outcomes. AE exposes casualties to reduced barometric pressure...
(hypobaria), as well as vibration, acceleration and temperature variations. The effects of hypobaria produced during AE have not been well studied in TBI patients.

Only a single published experimental study has examined the effects of hypobaria on TBI; using weight-drop induced brain injury in mice, exposure to 5h of hypobaria (8800ft) at 3h, but not 24h, after injury, increased neuron specific enolase (NSE)- a serum marker of neuronal injury- and cerebral levels of the inflammatory cytokine IL-6. Given such limited experimental data, there is a clear need to further characterize the effects of flight-equivalents on TBI, the mechanisms involved in potential exacerbation of injury, as well as the effects of timing of transport. In the present study we used a rat model of mild TBI to evaluate the effects of simulated AE on long term histological and behavioral outcomes in order to determine the “best time to fly” after a TBI. Our data indicate that exposure to prolonged hypobaria during the first hours to days following a TBI exacerbates pathophysiological changes, resulting in greater neurological deficits and related histological changes.
Materials and Methods

Animals

Adult male Sprague Dawley rats (Harlan Labs, Frederick, MD) weighing 325g (+25g) were utilized for these studies. Animals received a standard laboratory diet with food and water *ad libitum*. All procedures and experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee at the University of Maryland and the United States Air Force.

Micro-fluid Percussion and Hypobaric Animal Experiments

Rats were anesthetized with isoflurane (4% induction, 2% maintenance) and a 5 mm craniotomy was made over the left parietal cortex midway between lambda and bregma as previously described.\(^6\)\(^7\) Using our custom micro-fluid percussion (FP) device, a 1.5-1.9 atmosphere (atm) pressure was used to produce a mild injury with regard to neurologic and histologic deficits.\(^6\) Sham animals underwent the same procedures without injury. Hypobaria was induced using a steel cylindrical chamber with interior dimensions of 46 cm wide and 112 cm long equipped with internal temperature, oxygen, carbon dioxide, and pressure gauges and connected to a vacuum pump. Animals were placed into the chamber in their home cages with access to water and food to reduce stress from acclimation to the HB chamber. Multiple animals in various groups were randomly exposed simultaneously. The chamber was de-pressurized over 30 min to reach 568 mm Hg (=8000 ft. altitude) - approximating the cabin pressure during military AE with cruising altitudes of 30 – 40 thousand ft. To account for the mean oxygen saturation decrease of 5.5% experienced at this pressure, 28% O\(_2\) was continuously
delivered to the chamber to maintain $pO_2$ at sea level despite the drop in atmospheric pressure. Chamber gases were continuously monitored to validate concentration of $O_2$ delivered, as well as to verify that $CO_2$ was not accumulating in the chamber. At 5.5h of “flight” the chamber was re-pressurized over 30 min to 1 atm (765 mm Hg), the animals were then removed. Interior chamber temperature was monitored continuously and maintained at $22 \pm 2^\circ C$.

Pulse oximetry measurements of hemoglobin oxygen saturation (SpO2) were applied to two anesthetized rats to determine the relative blood oxygen saturation when rats were exposed to hypobaria under 100% compared to 28% oxygen. The rats were anesthetized with ketamine (70 mg/kg) plus xylazine (10 mg/kg) to obtain accurate and continuous measurements both in and out of the altitude champer. As expected, the anesthetized rats were slightly hypoxic (SpO2 = 85-89%) prior to entering the chamber. Depressurization occurred slowly over 20 min to an equivalent of 8000 ft. altitude with room air (21% oxygen) entering the chamber. The SpO2 remained in the range of 85-89% for at least 10 min after reaching the target altitude. The gas entering the chamber was then changed to 28% oxygen, resulting in a rise in SpO2 to between 90 and 94%. Ten minutes later, the gas was changed to 100% oxygen, resulting in a further increase in SpO2 to between 95 and 98%.

**Experimental Procedure**

Groups of animals received TBI or sham injury (craniotomy only) and were subjected to hypobaria for 6hr at at either 6h, 24h, 72h, or 7d following TBI (Fig. 1A). Additional groups were evaluated to determine the effects of both primary AE (6h of HB
at 24h after injury) and secondary AE (10h of HB at 72h after injury) following TBI (Fig. 1B) or the effects of breathing 100% inspired O$_2$ concentrations (hyperoxia) during AE.

The experimental timeline of 30d post-HB was chosen to be consistent and comparable to established procedures for accurate assessment of behavioral and histological outcomes following TBI.$^{6,7,8}$ All behavioral tests were conducted by an experimenter blinded to the experimental groups. Behavioral testing included Morris water maze tests for learning and memory (post-HB days 14-18), novel object recognition test for retention memory (post-HB day 21) and the forced swim test (post-HB day 26) for depressive-like behaviors. Brains were collected either at post-injury day 7 (n=6 for each group) for or post-HB day 30 for pathologic or immunohistochemical analysis (Sham n=9, Sham + HB n=9, TBI no HB n=19, TBI + HB @ 6h n=15, TBI + HB @ 24h n=14, TBI + HB @ 72h n=14, Two flights n=15, 100% O$_2$ n=15).

**Behavioral Testing**

Composite Neuroscore

Standardized motor scoring was performed at 1, 7, 14, 21 and 28 days after HB exposure. Motor function was evaluated using seven separate tests, each of which was scored using an ordinal scale ranging from zero (maximally impaired) to five (normal function). The tests included the ability to maintain position on an inclined plane in the vertical or two horizontal positions for 5s, forelimb flexion and forced lateral pulsion, as previously detailed.$^{6,9}$ Each of seven individual scores (vertical angle, right and left horizontal angle, right and left forelimb flexion, right and left lateral pulsion) were added to yield a composite neurological score ranging from 0 to 35.
Morris Water Maze

Spatial learning and memory were assessed using the acquisition paradigm of Morris Water Maze (MWM) test as previously described. A circular pool (1.5 m in diameter) was divided into four quadrants using computer-based AnyMaze video tracking system (Stoelting Co., Wood Dale, IL). Each rat was subjected to 4 trials to locate the hidden platform every day from post-HB days 14-17 (acquisition phase). Latency (seconds) to locate the hidden platform was measured, with a 90 second limit per trial, and swimming velocities assessed. Water maze search strategy analysis was also performed as previously described. Reference memory was assessed by a probe trial carried out on post-HB day 18. A visual cue test was also performed on post-HB day 18.

Novel Object Recognition

Non spatial retention and recognition memory was assessed by the novel object recognition test as previously described. On post-HB day 20 animals were placed into the open field and allowed to explore for 10 minutes each without any of the objects present for habituation and familiarization. On the testing day (post-HB day 21) two trials of 5 minutes each were performed. The first trial (training phase) involved placing identical square shaped “old objects” in both zones of the open field. The second trial (testing phase) involved placing one square shaped “old object” and one triangular shaped “novel object” in respective zones of the open field. The time that was spent exploring each object during both trials was recorded. In addition, time spent in novel object and old object zones was analyzed and compared between groups separately. The cognitive outcomes were calculated as the “Discrimination Index” (D.I.) for the second
trial using the following formula: \( \% \text{ D.I.} = (\text{Time spent exploring novel object/(Total time spent exploring both objects)}) \times 100 \).

Forced Swim Test

The forced swim test was used to examine depressive like behaviors. On post-HB day 26 rats were individually forced to swim inside a vertical plastic container (height: 60 cm; diameter: 25 cm) containing 30 cm of water for a time period of 6 minutes. The total duration of immobility (passive floating, slightly hunched, upright position, the head just above the surface) vs. struggle (diving, jumping, strongly moving all four limbs, scratching the walls) was recorded.

**Lesion Volume, Immunohistochemistry, and Quantification**

At 30 days post-HB rats were anesthetized with sodium pentobarbital (100 mg/kg, IP) and transcardially perfused with saline followed by 4% paraformaldehyde. Tissue was serially sectioned (60 \( \mu \text{m} \) or 20 \( \mu \text{m} \)) on a cryostat and mounted onto glass slides for histology and immunohistochemistry. Every eighth brain section (60 \( \mu \text{m} \)) beginning from a random start point was stained with cresyl violet (FD Neuro Technologies, Baltimore, MD) for quantification of lesion volume in the ipsilateral cortex based on the Cavalieri method of unbiased stereology using Stereoinvestigator software (MBF Biosciences, Williston, VT). Lesion volume was quantified by out-lining the missing tissue on the injured hemisphere using the Cavalieri estimator with a grid spacing of 0.1 mm, as previously described.

Standard fluorescent immunocytochemistry on 20-\( \mu \text{m} \) thick sections was performed as described previously. The following primary antibodies were used: rabbit
anti-Iba-1 (1:1000; Wako Chemicals, Richmond, VA, USA), mouse anti-CD68 (ED1, 1:500; AbDerotec, Raleigh, NC, USA), mouse anti-gp91phox (1:200; BD Transduction Laboratories, Franklin Lakes, NJ), mouse anti-GFAP (1:1000; Sigma). Fluorescent-conjugated secondary antibodies (Alexa 488-conjugated goat anti-mouse or rabbit, 1:1000; Molecular Probes, Grand Island, NY, USA) were incubated with tissue sections for 1h at room temperature. Cell nuclei were labeled with 4’, 6-diamidino-2-phenylindole (1 μg/ml; Sigma, St. Louis, MO, USA). Finally, slides were washed and mounted with an anti-fading medium (Invitrogen, Grand Island, NY, USA). Immunofluorescence microscopy was performed using a Leica TCS SP5 II Tunable Spectral Confocal microscope system (Leica Microsystems, Bannockburn, IL, USA). The images were processed using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA).

All immunohistological staining experiments were carried out with appropriate positive control tissue, as well as primary/secondary-only negative controls. For GFAP quantitative image analysis, digital images at 20× magnification were captured from the injured cerebral cortex and using a confocal laser-scanning microscope (n=3 sections/location/time point/rat for 5–6 rats/group). These were analyzed to quantify GFAP density and areas with ImageJ software (1.43; NIH, Bethesda, MD, USA).10

**Neuronal Survival in Hippocampal Sub-regions**

Brain sections stained with cresyl violet (n=4-10 rats/group) were used for assessment of neuronal cell loss using Stereoinvestigator software (MBF Biosciences, Williston, VT) as described previously.7,11 Total number of surviving neurons in the cornus ammonis (CA)1, CA2, CA3, and dentate gyrus (DG) subregions of the
hippocampus was assessed using the optical fractionator method. Every fourth 60 µm section between −2.04 mm and −4.56 mm from bregma was analyzed, beginning from a random start point. The volume of each hippocampal subfield was measured using the Cavalieri estimator method. The estimated number of surviving neurons in each field was divided by the volume of the region of interest to obtain the neuronal cellular density, expressed as counts/mm³.

**Stereological Quantification of Microglial Phenotypes in Ipsilateral Cortex**

Every fourth 60 µm brain section was immunostained for the microglia marker ionized calcium-binding adapter molecule 1 (IBA-1) and analyzed using a Leica DM4000B microscope (Leica Micro-systems Inc., Buffalo Grove, IL, USA). The number of cortical microglia in either activated or resting morphologic phenotypes were counted using the optical fractionator method with Stereoinvestigator software (MBF Biosciences) as described previously (n=5-12/group). The sampling region was between −2.04 mm and −4.56 mm from bregma in the ipsilateral cortex with a dorsal depth of 2.0 mm from the surface. The volume of the region of interest was measured using the Cavalieri estimator method. The estimated number of microglia in each phenotypic class was divided by the volume of the region of interest to obtain cellular density expressed in counts/mm³.

**Statistical Analysis**

Quantitative data were expressed as mean ± standard error of the mean (SEM). Functional data (latency to find the platform in seconds) for the acquisition phase of the
MWM were analyzed by repeated measure (trial over time) two-way ANOVA (injured/sham and hypobaria groups) to determine the interactions of post-injury days and groups, followed by post-hoc adjustments using the Student-Newman-Keuls test. The comparison of search strategies during the final day of trials of the MWM acquisition phase were analyzed using a chi-square test. The data for the probe trial for the MWM, the novel object recognition test, and the forced swim test were analyzed using a one-way ANOVA followed by post-hoc adjustments using the Student-Newman-Keuls test. In cases where only two groups were being compared a two-tailed Student’s t test was used for analysis. Assessment of motor function utilizing composite neurological scores were analyzed using the non-parametric Kruskal Wallis ANOVA followed by Dunn’s multiple comparison test. Stereological and lesion volume assessments were analyzed by one-way ANOVA followed by post-hoc adjustments using Student-Newman-Keuls test. All tests were performed using either SigmaPlot 12 (Systat Software; San Jose, CA) or GraphPad Prism program; Version 4.0 (GraphPad Software; San Diego, CA). A p value of less than 0.05 was considered statistically significant (* vs. sham injury; # vs. TBI no HB, ^ vs. TBI + HB).
Results

Hypobaria Worsens Cognitive Function following TBI.

To evaluate the long-term effects of HB exposure on TBI-induced neurological impairments motor and cognitive functional testing were conducted over one month post-HB. Spatial learning was tested using the acquisition phase of the MWM test to examine whether HB exposure following TBI increased deficits in hippocampal dependent learning (Fig. 2). The factors of “post-injury days” (F(3,283)=123.39; p<0.001) and “groups” (F(5,283)=8.15; p<0.001) were found to be significant. The interaction of “post-injury days X groups” (F(15,283)= 5.522; p<0.01; repeated measures two-way ANOVA) was significant. All groups receiving a TBI showed impairments in spatial learning in comparison to the sham injury group (Fig. 2A; p<0.05). The TBI + HB at 6h, TBI + HB at 24h, and TBI + HB at 72h groups showed significant impairment in spatial learning in comparison to the TBI/ no HB group (Fig. 2A; p<0.05).

A chi-square analysis was used to compare search strategies utilized by the animals to locate the hidden platform across groups and was found to be significant (Fig. 2B; p< 0.0001, χ² =135.7, df=10). HB-exposed animals displayed an inefficient search strategy relying less on a direct strategy (spatial) with increased circling activity (looping) around the edge of the maze. The sham injury group displayed a predominately spatial search strategy (81% spatial, 18% systematic, 1% looping). In the TBI no HB group utilization of the spatial search strategy (65%) was lower than that of the sham injury group with systematic (31%) and looping (4%) strategies increased. A further reduction in the percentage of trials using the spatial search strategy was observed in HB exposure groups with 35%, 38%, and 32% in the 6h, 24h, and 72h HB exposure post-injury groups,
respectively. For groups exposed to HB utilization of a spatial search strategy was highest (41%) and the instance of looping was lowest (8%) in the HB exposure at 7d after TBI group.

Spatial memory was assessed using the MWM probe trial on day 18 post-HB to determine if HB exposure following TBI increases hippocampal dependent memory deficits (Fig. 2C). Although an ANOVA was found to be significant (p=0.025), post-hoc comparisons did not show significant differences across HB groups (Fig 2C); therefore, these groups were combined to compare TBI plus HB exposure to TBI alone (Fig. 2D). TBI + HB exposed animals demonstrated a significant impairment in spatial memory in comparison to either sham injury or TBI no HB animals (Fig. 2D; p<0.05, one way ANOVA followed by Student-Newman-Keuls post-hoc test).

**Hypobaria Exposure following TBI causes Depressive-like Behavior, but does not affect Retention Memory and Motor Function.**

Retention memory was assessed by novel object recognition test on post-HB day 21 (Fig. 3A). Animals showed an equal preference for the two identical objects during the training phase. During the testing phase the sham injury group showed a marked increase in the time exploring the novel versus the familiar objects and the discrimination index increased from 43.6 ± 2.3% to 55.1% ± 2.6% (p=0.0029, two-tailed t-test). The TBI no HB and HB exposure at 6h, 24h, 72h, or 7d after injury groups had no or reduced preference for the novel object. No statistically significant differences in the discrimination index for the testing phase were observed across TBI groups (p=0.19, one-way ANOVA).
The forced swim test was used to examine whether HB exposure increases depressive-like behaviors following TBI (Fig. 3B). HB exposure at 6h, 24h, and 72h following TBI significantly increased depressive-like behavior vs. the sham injury group (p<0.001 TBI + HB @ 6h vs. sham injury; p<0.05 TBI + HB @24h vs. sham injury; p<0.01 TBI + HB @ 72h vs. sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test). HB exposure at 6h following injury significantly increased depressive-like behavior vs. TBI no HB group (p<0.01, TBI + HB @ 6h vs. TBI no HB; one-way ANOVA followed by Student-Newman-Keuls post hoc test).

On post-hypobaria day one and seven all groups receiving a TBI performed significantly worse on motor function assessment than the sham injury group with the exception of the TBI + HB @ 7d group on day one (Fig. 3C; p<0.001; non-parametric Kruskal Wallis ANOVA followed by Dunn’s multiple comparison test). On day 14 the TBI + HB @ 6h, 24h, and 7d groups scored significantly lower than the sham injury group (Fig. 3C; p<0.001; non-parametric Kruskal Wallis ANOVA followed by Dunn’s multiple comparison test). On day 21 the TBI + HB @ 24h and 72h groups scored significantly lower than the sham injury group (Fig. 3C; p<0.01). All groups recovered to sham injury levels by one month.

**Hypobaria Exposure does not worsen lesion volume following mild TBI.**

In order to determine if HB exposure following TBI increased lesion volume the Cavalieri method of unbiased stereology was used to assess brain sections on post-HB day 30 (Fig. 4A). Lesion volume was quantified by outlining the missing tissue on every eighth section of the injured hemisphere using the Cavalieri estimator (n=4 rats/group).
No significant difference in lesion volume was observed between groups (p=0.12; one-way ANOVA). TBI plus HB exposure groups (HB @ 6h, 24h, and 72h) were combined and compared to TBI animals not exposed to HB (TBI no HB group) in order to determine if there was an effect of HB exposure at anytime following TBI on lesion volume in comparison to no HB exposure following TBI (Fig. 4B). No significant difference in lesion volume was observed (p=0.18; two-tailed Student’s t-test).

**Hypobaria further worsens TBI-induced Neuronal Loss in the Hippocampus.**

Total neuronal cell numbers in the hippocampus were evaluated at post-HB day 30 to determine if deficits in spatial learning and memory correlated with neuronal cell loss (Fig. 5). Unbiased stereological quantifications show that mild TBI (represented by the TBI no HB group) caused significant neuronal cell loss in the CA1 and CA2/3 subregions of the hippocampus and the dentate gyrus vs. the sham injury group (Fig. 5A & D: Total & DG p<0.05; Fig. 5B & C; CA1 & CA2/3 p<0.01 TBI no HB vs. Sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test). HB exposure at 6h, 24h, or 72h following TBI significantly decreased the total number of hippocampal cells vs. the sham injury (Fig. 5A: p<0.001 TBI + HB @ 6h, 24h, 72h vs. Sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test) as well as the number of hippocampal cells in the CA1 (Fig. 5B: p<0.001 TBI + HB @ 6h, 24h, 72h vs. Sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test), CA2/3 (Fig. 5C: p<0.001 TBI + HB @ 6h, 24h, 72h vs. Sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test), and DG (Fig. 5D: p<0.01 TBI + HB @ 6h, 24h, 72h vs. Sham injury; one-way ANOVA followed by Student-
Newman-Keuls post hoc test). HB exposure at @ 6h, 24h, or 72h decreased the number of total cells surviving in the hippocampus in comparison to the TBI no HB group (Fig. 5A: p<0.01 TBI + HB @ 6h vs. TBI no HB; p<0.05 TBI + HB @ 24h or 72h vs. TBI no HB; one-way ANOVA followed by Student-Newman-Keuls post hoc test). HB exposure at @ 6h, 24h, or 72h increased hippocampal cell loss in both the CA1 and CA2/3 regions in comparison to the TBI no HB group (Fig. 5B & C: p<0.01 TBI + HB @ 6h vs. TBI no HB; p<0.05 TBI + HB @ 24h or 72h vs. TBI no HB; one-way ANOVA followed by Student-Newman-Keuls post hoc test).

**Hypobaria Exposure following TBI increases the glial response in the injured cortex.**

Using morphological phenotypes to differentiate between resting and activated states (Fig. 6), stereological quantification of microglial cell numbers in the injured cortex was performed at 7d post-injury (Fig. 6A) and at 30d post-injury (Fig. 6B) in order to examine whether an increased inflammatory response was associated with HB exposure. At 7d post-injury the number of activated microglial cells in the ipsilateral cortex were significantly increased in all TBI groups compared to the sham injury (p<0.05 TBI no HB, TBI + HB @ 24h & TBI + HB @ 72h vs. sham injury; p<0.01 TBI + HB @ 6h vs. sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test). At 30d post-HB the number of activated microglia remained significantly increased in the HB exposed groups in comparison to the sham injury (Fig. 6B; p<0.05 TBI + HB @ 24h & TBI + HB @ 7d vs. sham injury; p<0.001 TBI + HB @ 6h & TBI + HB @ 72h; one-way ANOVA followed by Student-Newman-Keuls post hoc test). The number of activated microglial at 30d post-HB also was significantly increased in the
TBI + HB @ 6h or 72h groups vs. TBI no HB (p<0.05; one-way ANOVA followed by Student-Newman-Keuls post hoc test).

In the Sham or Sham/HB brains, Iba-1, ED1, and gp91\textsuperscript{phox} immunoreactivity were weakly expressed. At 30 days after mild TBI, Iba-1, ED1, and gp91\textsuperscript{phox} immunoreactivity were up-regulated in the lesion area. Double-labeling immunohistochemistry revealed that many Iba-1\textsuperscript{+} microglia/macrophages were ED1 (Fig. 7A) or gp91\textsuperscript{phox} (Fig. 7B) positive after TBI. HB exposure at 6h increased the number of cells expressing Iba-1 and ED1 or gp91\textsuperscript{phox}.

In Sham or Sham/HB brains, GFAP immunoreactivity was relatively weak. At 30 days after mild TBI, GFAP immunoreactivity was up-regulated in the lesion area (Fig. 8A). Hypobaria exposure at 6h increased the GFAP\textsuperscript{+} astrocyte immunoreactivity. Quantification of pixel intensity for GFAP revealed significant astrocyte activation following HB at 6h after TBI (Fig. 8B-C).

**Effects of Hyperoxia or Simulated Primary and Secondary AE following TBI**

As supplemental oxygenation is routinely provided to TBI patients transported via AE the effects of 100% O\textsubscript{2} (hyperoxia) during HB following TBI on functional cognitive outcomes were evaluated. In addition the full course of transport for TBI patients, primary AE (6h of HB at 24h after injury) and secondary AE (10h of HB at 72h after injury) following TBI, was evaluated. The TBI + HB @ 6h, 24h, and 72h groups showed no statistical difference across groups for any tests in the first experiment. These groups (TBI + HB @ 6h, 24h, 72h) received 28% supplemental oxygen (normoxia) during a single HB exposure and were therefore combined to serve as a control for comparison to
animals receiving 100% supplemental oxygen (hyperoxia) during HB and to animals receiving both primary (HB @ 24h following TBI) and secondary AE (HB @ 72h following TBI) based on the similarity in the conditions and timing of HB exposure following TBI (TBI + HB n=43, TBI + 100% O₂ HB n=15; TBI + 1° & 2° HB n=15).

Spatial learning was tested using the acquisition phase of the MWM test to examine whether hyperoxia during HB exposure or two exposures to HB following TBI increases deficits in hippocampal dependent learning (Fig. 9A). The factor of “post-injury days” (F(3,201)=29.478; p<0.001) was found to be significant. The factor of “groups” (F(2,201)=0.739; p=0.48) was not significant. The interaction of “post-injury days X groups” (F(6,201)=0.48; p=0.82; repeated measures two-way ANOVA) was not significant. The results indicate that neither hyperoxia during HB exposure or two exposures to HB following TBI increased deficits in hippocampal dependent learning over that of a single HB exposure with normoxic oxygen levels following TBI.

A chi-square analysis was used to compare swimming pattern strategies during all trials on the fourth day of the acquisition phase across groups and was found to be significant (Fig. 9B; p< 0.05, χ² =9.98, df=4). Animals receiving either 100% O₂ during HB exposure or two HB exposures displayed a less efficient search strategy relying less on a direct strategy (spatial) than that of TBI + HB exposed animals. On day 18 post-HB spatial memory was assessed using the MWM probe trial by examining the total time spent in the target zone following removal of the platform to determine if hyperoxia during HB exposure or two exposures to HB following TBI increases hippocampal dependent memory deficits (Fig. 9C). Animals receiving either 100% O₂ during HB exposure or two HB exposures spent significantly less time in the target quadrant than
the TBI + HB group indicating an increased deficit in spatial memory (p<0.05, 100% O₂ vs. TBI + HB; p<0.05, 1° & 2° HB vs. TBI + HB: two-tailed Student’s t-test).

Effects of Hyperoxia or Primary and Secondary Evacuation on Retention Memory, Depression, and Motor Function.

Retention memory was assessed by novel object recognition test on post-HB day 21 (Fig. 10A). Animals showed an equal preference for the two identical objects during the training phase. No statistically significant differences in object preference during the testing phase or the discrimination index for the testing phase were observed between the TBI + HB group and either animals exposed to hyperoxia during HB or animals receiving two HB exposures following TBI (p=0.06 100% O₂ vs. TBI + HB; p=0.48, 1° & 2° HB vs. TBI + HB: two-tailed Student’s t-test).

The forced swim test was used to evaluate increases in depressive-like behaviors on post-HB day 26 (Fig. 10B). No significant differences were observed in depressive like behaviors between the TBI + HB group and either animals exposed to hyperoxia during HB or animals receiving two HB exposures following TBI (p=0.08 100% O₂ vs. TBI + HB; p=0.68, 1° & 2° HB vs. TBI + HB: two-tailed Student’s t-test).

Motor function was assessed as previously described. A non-parametric Kruskal Wallis ANOVA was used to analze differences across groups for each time point. There were no significant differences in motor scoring between TBI + HB group and either animals exposed to hyperoxia during HB or animals receiving two HB exposures following TBI (Fig. 10C; Day 1 p=0.73; Day 7 p= 0.763; Day 14 p= 0.65; Day 21 p= 0.87; Day 28 p= 0.07).
Discussion

Although there have been clinical impressions that the stressors and hypobaria (HB) associated with AE may exacerbate traumatic brain injury (TBI), there have been no clinical studies and only a single limited animal study to address this issue.\(^5\) In the latter study, exposure to HB at 3h, but not 24h following induction of TBI, increased levels of the inflammatory cytokine IL-6 and serum neuron specific enolase. There were key differences between this report and the current study, including injury model and species. Goodman et al used a mouse controlled cortical impact model, in contrast to our rat LFP model. Other differences include HB exposure initiation time, and higher simulated altitude (8800ft) over a shorter duration (5h) than in the current study (8000ft/6h). But perhaps the most important difference between these studies relates to levels of oxygenation during HB exposure. Goodman et al provided no supplemental oxygen and measurements of oxygen saturation levels demonstrated significant hypoxia in HB exposed animals. However, in the current study 28% \(O_2\) was continuously delivered to maintain normoxia, in order to separate effects of HB exposure from hypoxia which is known to worsen TBI outcome.\(^{12}\)

This study is the first to examine the effects of HB, as occurs during AE, on long-term behavioral outcomes following experimental TBI, as well as the effects of HB simulating AE without the confounding effects of hypoxia. The duration of HB exposure chosen (6h) was based on the average AE flight time between combat support hospitals and non-theater hospitals during the Operation Iraqi Freedom and Operation Enduring Freedom conflicts. The data provides evidence to suggest that exposure to prolonged HB
within 7 days following injury may exacerbate cognitive deficits associated with increased neuroinflammation and secondary brain injury.

**Influence and Timing of AE following TBI on Cognitive Function and Depression-like Behavior**

Due to potential of HB exposure exacerbating injury following TBI, behavioral testing began at fixed times after HB exposure rather than following TBI to insure that all behavioral testing occurred during a similar time frame following the last insult. Differences in the timing of HB exposure during the first three days following injury (6h, 24h, 72h) did not significantly alter the negative impact of HB on behavioral outcomes. Although HB exposure at 7d following injury appeared to produce less exacerbation on some tests, it was not found to be statistically different from HB exposure at other time points. The difference in timing of behavioral tests following injury induction among HB groups is not believed to have affected cognitive outcomes, as the chronic neuronal damage caused by TBI is slowly progressive after the first week.

Patient transport typically requires a 6-8h flight within the first 24-48 hours following injury followed within 4-5 days of injury by an intercontinental flight averaging 10 hours to a medical center in the United States for definitive care. Therefore, the effects of two HB exposures (simulating both primary and secondary AE) on TBI animals was evaluated. Comparison of the effects of single versus two sequential HB exposures showed increased spatial memory deficits in the latter group. Due to the apparent additive effect of multiple HB exposures following TBI, further evaluation on the timing of secondary transport is warranted.
TBI patients are routinely provided with 70-100% oxygen during transport. In the present study hyperoxia resulting from 100% oxygen treatment during HB exposure following TBI increased cognitive deficits in the Morris water maze in comparison to normoxic conditions. The effects of hyperoxia alone following TBI in the absence of HB were not evaluated in this study, therefore we cannot conclude whether the increase in cognitive deficits are due to hyperoxia alone or the combination of HB and hyperoxia. However, the results of this experiment and other evidence suggests that oxygen supplementation following TBI may be damaging.14,15 Two recent retrospective clinical studies evaluating the effects of hyperoxia within the first 24h following brain injury show that hyperoxic conditions are associated with worse functional outcomes and increased mortality.16,17 Evidence that the oxygen level present within the altitude chamber affected the systemic levels of oxygen during exposure to hypobaria came from additional experiments utilizing pulse oximetry (See Materials and Methods). A pulse oximeter was placed on the neck of two different rats anesthetized with ketamine plus xylazine. The rats were placed in the altitude chamber and measurements made of the hemoglobin oxygen saturation (SpO2) present 20 min later under hypobaric conditions with 28% oxygen entering the chamber. The SpO2 varied within a range of 90 to 94%, indicative of normoxia. Ten minutes later, the level of ambient oxygen was elevated to 100%, resulting in SpO2 values between 95 and 98%. Further investigation into optimizing levels of oxygen supplementation during HB exposure is warranted.

The increased cognitive deficits resulting from HB exposure, using the classic Morris Water Maze (MWM) evaluation, were relatively modest. In part, this may reflect the substantial cognitive deficits induced by lateral fluid percussion alone, which required
titrating toward lower injury to limit potential “ceiling” effects. For this reason, a more sophisticated analysis was used in addition to traditional evaluations of performance. Examination of navigational search strategies on the final day of the acquisition trials can be helpful in further determining behavioral differences between groups that show similar escape latencies.\textsuperscript{7,11} The underlying differences in search strategy showed that injured animals not exposed to HB used predominantly spatial strategies, similar to that of sham-injured animals. Injured animals exposed to HB displayed reduced spatial learning abilities, indicated by their reliance on a less efficient systematic search strategy or use of non-spatial “looping” behavior. No significant differences in motor function were detected between TBI groups with or without HB exposure, indicating that motor dysfunction was not a contributing factor to deficits observed during cognitive testing.

Impaired cognitive performance in the MWM task is an indicator of hippocampal damage. The relative contribution of various hippocampal sub-regions in encoding and retrieval of spatial learning and memory can be quantified by counting neurons in such sub-regions.\textsuperscript{7,18} Loss of neurons in the CA3 and DG sub-regions is associated with cognitive impairment in spatial navigation and object exploration tasks. Data here show that HB exposure following injury causes a significantly greater loss of neurons in CA1/2/3, with only modest differences in the dentate gyrus, compared to injured control animals. This loss of neuronal hippocampal cells is consistent with the impairments in spatial learning and memory caused by HB exposure. The novel object recognition test evaluates retention memory and correlates less with hippocampal changes;\textsuperscript{19} in this test HB exposed animals displayed no significant differences during the testing phase.
Depression is common in patients after TBI and can negatively affect recovery.\textsuperscript{20} The forced swim test was used to examine depressive-like behaviors.\textsuperscript{21} HB exposure at 6h following TBI increased depressive-like behaviors in comparison to injured animals not exposed to HB.

\textbf{Influence of AE following TBI on Neuroinflammation}

Microglial activation has been recognized as a key contributor to secondary injury severity and progressive cognitive decline following TBI.\textsuperscript{22} Areas of neuronal degeneration and axonal abnormality frequently correspond to sites of microglial activation.\textsuperscript{23,24} Sustained microglial activation is associated with changes in cognitive function.\textsuperscript{25} Microglial activation has been shown to persist for at least 12 months following TBI in non-human primates.\textsuperscript{26} Post mortem studies in humans show persistent elevated microglial activity for years after TBI.\textsuperscript{27,28,29} Moreover, a clinical study using positron emission tomography, has shown persistent microglial activation many years after TBI in humans, particularly in areas distant from the initial trauma site and associated with cognitive impairment.\textsuperscript{30} The present data demonstrated that HB exposure following TBI caused increases in the number of activated microglia, which were significantly elevated at 30d post-HB in comparison to injured controls. As long-term microglial activation has been shown to persist for years and and likely contribute to progressive cognitive decline,\textsuperscript{25,30} the increased activation of microglia following TBI and AE may contribute the development of chronic neurodegeneration.

\textbf{Conclusion}
The present study demonstrates that HB exposure during simulated air transport following TBI exacerbates neuroinflammatory secondary injury mechanisms, leading to increased deficits in learning and memory, as well as hippocampal neuronal cell loss. The present studies suggest possibilities for mitigating injury exacerbations by HB exposure following TBI, including retaining patients at in-theater hospitals for longer periods prior to transport; delaying the second AE; increasing cabin pressurization to reduce barometric effects or specialized enclosures for individual pressurization; and/or changes to the supplemental oxygenation protocols.
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**Figure Legends:**
A. Groups received TBI or sham injury and were subjected to hypobaria for 6hr at either 6h, 24h, 72h, or 7d following TBI.

B. Groups received TBI and were subjected to an initial period of hypobaria for 6h at 24h after injury and a second hypobaria exposure for 10h at 72h after injury.

Figure 1. Experimental Timeline for Evaluating the Effects Hypobaria following TBI.
A. TBI caused significant deficits in spatial learning and memory function in the acquisition phase of MWM in the TBI no HB and TBI + HB groups compared to sham injury (*p<0.05 vs. sham. HB exposure between 6h-72h following TBI further exacerbated learning deficits in comparison to the TBI no HB group (#p<0.05 vs. TBI no HB).

B. HB-exposed animals were less efficient in their search strategy on the fourth day of the acquisition phase while attempting to locate the hidden platform relying less on a direct strategy (spatial) and an increase in the amount of circling activity (looping) chi-square analysis (p< 0.0001, $\chi^2=135.7$, df=10).
C. Spatial memory was assessed using the MWM probe trial on day 18 post-HB. Post-hoc comparisons did not differ significantly across groups.

D. TBI plus HB exposure groups (HB @ 6h, 24h, 72h, or 7d) did not significantly differ and were combined to determine if there was an effect of HB exposure at anytime following TBI on spatial memory in comparison to no HB exposure following TBI. TBI + HB exposed animals demonstrated a significant impairment in spatial memory in comparison to TBI no HB animals (*p<0.05 vs. sham injury; # p<0.05 vs. TBI no HB).
Figure 3. Effects of Hypobaria Exposure following TBI on Retention Memory, Depression and Motor Function.

A. Retention memory was assessed by novel object recognition test on post-HB day 21. During the testing phase the sham injury group showed a marked increase in the time exploring the novel versus the familiar objects (p=0.0029, two-tailed t-test). No statistically significant differences in the discrimination index for the testing phase were observed across TBI groups (p=0.1878, one-way ANOVA).

B. The effect of HB exposure following TBI on depressive-like behaviors was assessed of post-HB day 26 using the forced swim test. HB exposure at 6h, 24h, or 72h following TBI significantly increased depressive-like behavior vs. the sham injury group (**p<0.001 TBI + HB @ 6h vs. sham injury, *p<0.05 TBI +
HB @24h vs. sham injury; **p<0.01 TBI + HB @ 72h vs. sham injury; one-way ANOVA followed by Student-Newman-Keuls post hoc test). HB exposure at 6h following injury significantly increased depressive-like behavior vs. TBI no HB group (##p<0.01, TBI + HB @ 6h vs. TBI no HB).

C. Motor function was assessed on post-HB days 1, 7, 14, 21, and 28 using a composite assessment of established tests. Animals receiving a TBI returned to sham injury levels within one month, with no differences across TBI groups.
Figure 4. Effects of Hypobaria Exposure following TBI on Lesion Volume.

A. Lesion volume was assessed at post-HB day 30 using the Cavalieri method of unbiased stereology. No significant difference in lesion volume was observed between groups (p=0.12; one-way ANOVA).

B. TBI plus HB exposure groups (HB @ 6h, 24h, and 72h) were combined and compared to TBI animals not exposed to HB (TBI no HB group) in order to determine if there was an effect of HB exposure following TBI on lesion volume in comparison to no HB exposure following TBI. No significant difference in lesion volume was observed (p=0.18; two-tailed Student’s t-test).
Figure 5. Hypobaria following TBI Increases Neuronal Loss in the Hippocampus.

Total neuronal cell numbers in the hippocampus were evaluated at post-HB day 30 to determine if deficits in spatial learning and memory correlated with neuronal cell loss. Unbiased stereological quantifications show significant neuronal cell loss in the CA1 and CA2/3 subregions of the hippocampus and the dentate gyrus in the TBI no HB group in comparison to the sham injury group that (Total & DG *p<0.05; CA1 & CA2/3 **p<0.01). HB exposure at @ 6h, 24h, or 72h decreased the number of total cells surviving in the hippocampus in comparison to the TBI no HB group (##p<0.01 TBI +
HB @ 6h vs. TBI no HB; #p<0.05 TBI + HB @ 24h or 72h vs. TBI no HB). HB exposure at @ 6h, 24h, or 72h increased hippocampal cell loss in both the CA1 and CA2/3 regions in comparison to the TBI no HB group (##p<0.01 TBI + HB @ 6h vs. TBI no HB; #p<0.05 TBI + HB @ 24h or 72h vs. TBI no HB).
The number of activated microglial cells in the ipsilateral cortex 7d post-injury was significantly increased in the TBI groups compared to the sham injury group (*p<0.05, **p<0.01 vs. sham). At 30d post-HB the number of activated microglial cells remained significantly elevated in all TBI + HB exposed groups in comparison to the sham injury group (*p<0.05, **p<0.01, ***p<0.001 vs. sham). The number of activated microglial cells also remained significantly elevated in the TBI + HB @ 6h and 72h groups in comparison to the TBI no HB group (#p<0.05 vs. TBI no HB).
Figure 7. TBI-induced immunoreactivity of Iba-1, ED1, and gp91\textsuperscript{PHOX} was increased at the injured cortex by 6 hours hypobaria (HB) exposure. In the Sham or Sham/HB brains, Iba-1, ED1, and gp91\textsuperscript{PHOX} immunoreactivity were weakly expressed. At 30 days after TBI, Iba-1, ED1, and gp91\textsuperscript{PHOX} immunoreactivity were up-regulated in the lesion area. Many Iba-1\textsuperscript{+} microglia/macrophages were ED1 (A) or gp91\textsuperscript{PHOX} (B) positive after TBI. HB exposure at 6h increased the positive cell numbers of Iba-1, ED1, and gp91\textsuperscript{PHOX} cells. Scale bar = 100 \(\mu\text{m}\).
Figure 8. Hypobaria exposure (HB) @ 6h increased astrogliosis at the injured cortex following TBI. (A) In the Sham or Sham/HB brains, GFAP immunoreactivity was relatively weak. At 30 days after mild TBI, GFAP immunoreactivity was up-regulated in the lesion area. Hypobaria exposure at 6h increased the GFAP+ astrocyte immunoreactivity. Scale bar = 100 μm. (B-C) Significantly increased GFAP activity was found at 6h HB after TBI. N=5–6 rats/group. ***p<0.001 vs Sham/HB control; #p<0.01, ###p<0.001 vs TBI group.
Figure 9. Cognitive Assessment following Hyperoxia or Primary and Secondary Evacuation.

A. No further decrement in spatial learning was observed in the acquisition phase of the MWM test following hyperoxia during HB exposure or two exposures to HB following TBI. B. Animals receiving 100% O₂ during HB exposure or two exposures to HB following TBI displayed a less efficient search strategy on the fourth day of the acquisition phase in comparison to animals receiving normoxic conditions during a single HB exposure relying less on a direct spatial strategy (p< 0.05, χ² = 9.981, df=4; chi-square analysis). C. Animals receiving either 100% O₂ during HB exposure or two HB exposures spent significantly less time in the target quadrant than the TBI + HB group.
indicating an increased deficit in spatial memory (\(^p<0.05, 100\% \text{ O}_2 \text{ vs. TBI + HB};\) 
\(^p<0.05, 1^\circ \text{ & } 2^\circ \text{ HB vs. TBI + HB: two-tailed Student’s t-test).\)

**Figure 10. Effects of Hyperoxia or Primary and Secondary Evacuation on Retention Memory, Depression, and Motor Function.**

A. Non-spatial memory was assessed by novel object recognition test on post-HB day 21. Animals showed an equal preference for the two identical objects during the training phase. No statistically significant differences in the discrimination index for the testing phase were observed across groups (\(p=0.48 100\% \text{ O}_2 \text{ vs. TBI + HB}; p=0.06, 1^\circ \text{ & } 2^\circ \text{ HB vs. TBI + HB: two-tailed Student’s t-test).\) B. The forced swim test was used to assess depressive-like behaviors on post-HB day 26. No significant differences were observed in depressive like behaviors between groups (\(p=0.08 100\% \text{ O}_2 \text{ vs. TBI + HB}; p=0.68, 1^\circ \) & \(2^\circ \text{ HB vs. TBI + HB: two-tailed Student’s t-test).\) C. Motor function was assessed on post-HB days 1,7,14,21, and 28 using a composite assessment of established tests. No differences in motor function were observed between groups.