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**MAKING TACTICAL PRACTICAL – PRAGMATIC SOLUTIONS
TO PREVENTING NEUROLOGIC EFFECTS OF EARLY
AEROMEDICAL EVACUATION (AE) IN THE HEAD INJURED
PATIENT**

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ABSTRACT

Early aeromedical evacuation (AE) after traumatic brain injury (TBI) has been associated with worse neurologic outcomes. No potential therapeutics have been evaluated to prevent secondary injury induced by the hypobaric hypoxia (HHH) environment integral to post-injury AE. This study examined the role of allopurinol/propranolol/tranexamic acid

(TXA)/adenosine/lidocaine/magnesium (ALM)/amitriptyline administration prior to simulated flight. **Methods:** Murine and porcine models were utilized. *Murine* models underwent TBI and were given allopurinol/propranolol/amitriptyline/ALM prior to simulated AE or normobaric normoxia (NN) control. Heart rate (HR), respiratory rate (RR), and oxygen saturation (SpO₂) were recorded. Mice were euthanized at 24 hours/7 days/30 days. Serum and cerebral cytokines were assessed by ELISA. Motor function testing was performed with Rotarod ambulation. Immunohistochemistry was conducted to examine phosphorylated tau (p-tau) accumulation in the hippocampus. *Porcine* TBI was induced via controlled cortical injury. Survival and non-survival cohorts were separated into four groups: TBI+25mL normal saline (NS), TBI+4mg propranolol, TBI+100mg allopurinol, and TBI+1g TXA. Pigs then underwent simulated AE (8,000 ft) for 4 hours with an SpO₂ of 82-85% and were euthanized 4 hours later. Neurologic severity score, serum cytokines, and hippocampal p-tau accumulation were assessed. **Results:** *Murine* treatments improved SpO₂, propranolol, amitriptyline, and allopurinol improved AE-induced tachycardia. At 24 hours, both propranolol and amitriptyline reduced TNF- α levels while allopurinol and ALM reduced TNF- α levels only in NN mice. Propranolol, amitriptyline, and ALM demonstrated lower serum MCP-1 7 days after AE. Both amitriptyline and allopurinol improved Rotarod times for AE, while only allopurinol improved Rotarod times for NN mice. Propranolol was able to reduce p-tau accumulation under both HH and NN conditions while ALM only reduced p-tau in hypobaric hypoxic conditions. *Porcine* There were no significant differences in hemodynamics, tissue oxygenation, cerebral blood flow or physiologic markers between treatment groups and saline controls. Transient differences in IL-1 β and IL-6 were noted but did not persist. NSS was significantly lower in the TBI+allopurinol group on POD 1 compared to NS and propranolol groups. P-tau accumulation was decreased in the non-survival animals treated with allopurinol and TXA compared to NS and sham groups. **Conclusion:** *Murine* Propranolol lowered post-TBI HR with reduced pro-inflammatory effects, including p-tau reduction. Amitriptyline induced lower post-TBI HR and improved functional outcomes without affecting inflammatory response. Allopurinol did not affect vital signs but improved late post-TBI systemic inflammation and functional outcomes. ALM provided no short-term improvements but reduced p-tau accumulation at 30 days in the HH cohort. Allopurinol may be the best of the four treatments to help prevent short-term functional deficits while propranolol may address long-term effects. *Porcine* Allopurinol, propranolol, or TXA following TBI does not induce adverse change in systemic or cerebral hemodynamics during or after simulated post-injury flight. While transient changes were noted in systemic cytokines and p-tau accumulation, further investigation will be needed to determine any persistent neurological effects of injury, flight, and pharmacologic treatment.

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1.0 BACKGROUND:

Traumatic Brain Injury (TBI) has been the “signature injury” of American foreign military operations over the last two decades, with over 400,000 diagnoses of TBI since 2000¹. This incidence translates to approximately 11-23 percent (%) of deployed personnel experiencing some form of TBI². TBI consists of a primary injury which is caused by physical damage to the brain at time of impact followed by a secondary injury. These injuries are compounded by the fact that they typically occur in austere environments requiring AE, with an environment in which patients may be subjected to hypoxia, hypotension, and hypercarbia (HHH) during the hours (h) to days following the initial insult^{3,4}. Exposure to HHH from flight can create a second physiological insult, which may exacerbate the initial injury and lead to inferior outcomes.

Previous studies have demonstrated an increase in systemic and cerebral inflammation from HHH following TBI⁵⁻⁹. These conditions are inherent to the environment encountered during early AE after injury en route to definitive hospital care⁶. In a civilian prospective multicenter trial investigating pre-hospital care, mortality after TBI alone was found to be 20%, however, patients who also suffered a hypoxic episode had a mortality rate of 37%.¹⁰ The effects of HHH are not limited to mortality. A retrospective review of 438 patients who underwent AE after TBI demonstrated that delayed evacuation was associated with a lower mortality rate and a higher likelihood of discharge to home compared to patients who underwent early transport¹¹. These clinical results have also been supported histologically in animal studies that have demonstrated that rodents and swine experience worse tissue injury in the brain, lungs, small intestine, and kidneys compared to animals that did not undergo AE^{12,13}. In the non-military population, hypoxia has also been linked to the formation of amyloid precursor within the brain as well as the phosphorylation of tau protein, thereby increasing the risk of Alzheimer’s disease¹⁴⁻¹⁶.

However, delayed transport is not always feasible, either from a military mission tactical standpoint or due to the nature of the patients' other injuries.

Although the potential secondary brain injury induced by AE has been recognized, there has been limited investigation into potential therapeutics to prevent these detrimental effects of post-TBI AE. Clinically utilized medications following the physiologic insults of injury, hemorrhage or ischemia include propranolol, allopurinol, and tranexamic acid (TXA), adenosine/lidocaine/magnesium (ALM), and amitriptyline. Multiple clinical studies have shown improvement in 30-day mortality and clinical neurological outcomes with propranolol administration following TBI¹⁷⁻²¹. Propranolol, a nonselective beta-blocker, has been shown to consistently decrease mortality rate as well as decreasing hospital length of care and duration of intensive care if administered early after TBI²²⁻²⁴ and also shown to decrease neuroinflammation and p-tau accumulation within the hippocampus via multiple animal models^{5,25-28}. Allopurinol, a xanthine oxidase inhibitor that reduces the production of free radicals, has been shown to reduce brain edema acutely after hypoxic injury in a rodent model while improving evidence of morphological damage over time^{29,30}. TXA, an antifibrinolytic, has been studied extensively in TBI populations demonstrating improved short-term morbidity and head-injury associated mortality³¹⁻³⁵. Amitriptyline has been shown to improve symptoms after concussive injuries in humans and reduce hippocampal p-tau accumulation in a murine TBI model³⁶⁻³⁹. ALM has been shown to decrease vascular resistance to improve cerebral blood flow after hemorrhage in a porcine model⁴⁰. A rodent model has also shown that ALM reduces coagulopathy and inflammation after blunt TBI⁴¹.

Although these medications are routinely administered in the inpatient setting following TBI, none of these medications have been directly compared following TBI or evaluated in the setting of early post-injury administration prior to AE or the HHH environment.

This study main objective was to evaluate if commonly utilized medications including propranolol, allopurinol, and TXA), ALM, and amitriptyline could mitigate the secondary inflammation experienced exposure to HHH environment of AE using murine and porcine models.

2.0 METHODS:

Murine Model:

Utilized an established murine model of TBI and AE in which the mice received allopurinol, propranolol, ALM, or amitriptyline, prior to simulated flight following TBI. We subsequently evaluated vital signs in flight, acute inflammatory biomarkers, functional outcomes via Rotarod, as well as 30-day changes in phosphorylated tau (p-tau) protein concentration. We hypothesized that these drugs, particularly propranolol, would show an additional benefit in the AE environment.

Animal and Injury Model

The University of Cincinnati Institutional Animal Care and Use Committee and the United States Air Force Medical Support Agency Office of Research Oversight and Compliance, approved all animal experiments. All animals were 8–10-week-old male C57BL6/J mice acquired from Jackson Laboratory (Bar Harbor, Maine). Male mice were utilized in all experiments for consistency and to limit the recognized neuroprotective effects of estrogen and progesterone⁴². All mice were acclimated for one week prior to experimentation in a controlled housing environment and were provided food and water ad lib.

Our previously described weight drop model was used to induce a moderate TBI⁴³⁻⁴⁵. Briefly, mice were anesthetized with 2% inhaled isoflurane in 100% oxygen at 1 liter per minute (min) (L/min) for approximately 2 min. After an appropriate level of anesthesia was obtained, mice were placed in the prone position on a platform. The head was centered under a 400-gram cylindrical rod that was dropped from 2.5 centimeters (cm) above the surface of the table to induce a global concussive blunt TBI. All mice were observed to confirm appropriate respiratory and immediate neurologic recovery by righting from a post-TBI supine position. Sham TBI mice were anesthetized and positioned similarly but were not subjected to TBI.

Pharmacologic Intervention and Simulated Flight

Two and a half h following injury, mice were given 1 of 4 drug treatments (allopurinol [20 meter per kilogram (m/kg)], amitriptyline [10 milligrams per kilogram (mg/kg)], propranolol [4 mg/kg], or 0.7 microliter per gram (uL/g) of adenosine [1 Millimolar (mM)] / lidocaine [3 mM] / magnesium [2.4 mM]) or saline via intravenous injection. Sham TBI mice only received saline injections. This time point was chosen from previous studies that demonstrated secondary injury from simulated AE at 3 h after TBI ⁴⁶. A Mouse Ox Plus collar (Starr Life Sciences Corporation, Oakmont, PA) was placed to continuously monitor heart rate (HR), oxygen saturations (SpO₂), and respiratory rate (RR) of the mice. At 3 h post-injury, mice were exposed to normobaric normoxia (NN) to simulate ground conditions or HHH) to simulate AE for two h. Flights simulated an altitude of 8,000 feet (ft) using a custom-built altitude chamber located in the Center for Surgical Innovation at the University of Cincinnati (Abbess Instruments and Systems, Ashland, MA).

Cytokine analysis

Each group contained mice that were euthanized at 6 h, 24 h, or 7 days (n=5 per group at each timepoint) for serum and brain tissue cytokine analysis. Mice were anesthetized with pentobarbital 1 milligram per milliliter (mg/ml) via intraperitoneal injection and whole blood was obtained via cardiac puncture. Whole blood was placed in serum separator tubes (BD Bioscience, San Diego, California), centrifuged at 1,000 gram(s) (g) for 10 min, and the serum was collected. The brain of each mouse was obtained via craniectomy following euthanasia. All samples were stored at -80 degrees Celsius(°C) until analysis. Serum was analyzed by multiplex enzyme-linked immunosorbent assay (ELISA), for cytokines and chemokines including interleukin (IL) 1 alpha (IL-1 α), IL-1 beta (IL-1 β), IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p70, IL-17, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF α), macrophage inflammatory protein 1 alpha (MIP-1 α), granulocyte-macrophage colony-stimulated factor (GM-CSF), and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) (Quansys Biosciences, Logan, UT). Serum neuron specific enolase (NSE) was additionally analyzed by ELISA to assess this systemic biomarker of acute cerebral injury (IBL

America, Minneapolis, MN). Brain tissue was similarly analyzed by multiplex ELISA for cytokines and chemokines (Quansys Biosciences, Logan, UT). All tissue samples underwent analysis by bicinchoninic acid (BCA) (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific, Rockford, IL) to normalize cerebral protein content. These cytokines were chosen as they are not only critical to the pro-inflammatory pathway after TBI that is exacerbated by secondary injury but also to provide a comprehensive post-injury profile.

Motor Evaluation

The Rotarod device (IITC Life Science, Woodland Hills, CA) was utilized to assess sensorimotor coordination and motor learning⁴⁷⁻⁴⁹. Mice were tested the day prior to injury, post-injury day 2, and post-injury day 7 (n=5 per group). All mice were acclimated at a steady rate of 4 rotations per min for 60 seconds prior to each testing evaluation. Following acclimation, the rod was started at 4 rotations per min and accelerated to 45 rotations per min over a 3-min period. Each trial on the rod was terminated when the animal fell off the rod, held on to the rod and completed two complete revolutions, or remained on the rod for 600 seconds. Mice underwent three trials each day and the average trial time was calculated. A ratio of post-injury performance to pre-injury performance was also calculated for each mouse.

Histological Assessment of Phosphorylated Tau Protein (p-tau) Accumulation

To assess for p-tau accumulation, mice were survived to 30 days after injury. They were anesthetized with pentobarbital 1mg/ml via intraperitoneal injection and were perfused with 10% formalin via cardiac puncture. Brains were removed and fixed in 10% formalin for 48 h, dehydrated, and embedded in paraffin for immunohistochemistry. Coronal sections were taken as 5um slices. As previously described, they were stained with rabbit polyclonal antitau (phosphor S262) antibody (ab131354; Abcam, Cambridge, UK)³⁹. Images were captured on Nikon AIR GaAsP inverted microscope (Nikon Corporation, Tokyo, Japan) and quantified using the cell counter feature on ImageJ software.

Statistical analysis

All statistical analyses were performed with Prism 6 (GraphPad Software, La Jolla, California). Data are presented as the mean plus or minus ($\pm$) standard deviation or median (interquartile range). Mann-Whitney test was used to compare continuous variables between two groups. The Kruskal-Wallis test was used to compare continuous data between groups of three or more. T-tests were used to compare NN versus HHH response for each drug. A power (p) value < 0.05 was considered significant.

Porcine

The relevance of the above results in humans remains to be determined as the murine brain is much smaller and lissencephalic compared to the gyriiform brain of larger mammals⁵⁰. The goal of these experiments was to determine if commonly utilized medications including allopurinol, propranolol, or TXA could mitigate the secondary inflammation experienced during the hypobaric and hypoxic environment of AE using a porcine model. Based on previous clinical and preclinical data, we hypothesized that propranolol would demonstrate the most benefit in decreasing systemic inflammation as well as mitigating the phosphorylation of tau protein within the hippocampus.

Animal Model

This study was reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee as well as the United States Air Force Medical Support Agency Office of Research Oversight and Compliance. Animals were cared for by a program 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. Female Yorkshire pigs weighing 40 ± 2 kilograms (kg) were obtained from Isler Genetics (Prospect, OH) and were acclimated in our animal facility for 48-72 h before experimentation. Animals were housed alone or in pairs and provided with food and water without restriction, except for 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). Sedated pigs were placed in a supine position and orotracheally intubated. Pigs were maintained on a ventilator (Ohmeda, Madison, WI) in pressure control mode during non-altitude portions of the experiment and transferred to an Impact 731 Series Ventilator (IMPACT Instrumentation, West Caldwell, NJ) for simulated AE due to approved performance at altitude for these ventilators.

Porcine TBI Model

A TBI of moderate severity was induced through an established porcine model of controlled cortical injury (CCI)⁵¹. Catheters were placed via surgical cutdown approach in the femoral artery and internal jugular vein prior to positioning the animal in a stereotactic frame (Stoelting Co, Wood Dale, IL) in the prone position. An incision was then made in the midline of the scalp exposing the full length of the cranium including the lambdoid and coronal sutures. A 2 cm craniotomy was made 16 millimeters (mm) anterior to the coronal suture and 12 mm lateral to the sagittal suture leaving the dura intact. A contralateral 5.3 mm craniotomy was created for placement of a quad-lumen bolt (Hemedex, Cambridge, MA) and neuromonitoring devices for non-survival animals. Intracranial monitoring consisted of brain tissue oxygenation (Licox probe Integra, Saint Priest, France) and brain perfusion (Bowman Perfusion probe, Hemedex, Cambridge, MA). Moderate TBI was induced through the right craniotomy with a 15 mm wide controlled cortical impactor (Hatteras Instruments, Cary, NC) at 4 meter per second (m/s), 100 m/s dwell time and 13 mm depth onto the exposed dura and underlying cerebral parenchyma⁵¹. Following TBI, the craniotomy was not repaired, however skin was reapproximated over top using non-absorbable suture. Sham TBI animals for immunohistochemistry analysis underwent vascular cannulation and craniotomy but no cortical injury was induced.

Following TBI, swine were recovered for 1 h prior to administration of study medication. Non-survival animals were randomized into four groups of five animals each: TBI+25 milliliter (mL) normal saline (NS), TBI+4 milligram(s) (mg) propranolol, TBI+100mg allopurinol, and TBI+1g TXA, all via intravenous infusion. Survival animals were initially allocated into similar groups; however, this arm of

the study was terminated early and without the TXA group due to Coronavirus disease (COVID)-induced staffing restrictions. Simulated AE was then initiated to an altitude of 8,000 ft for 4 h via an altitude chamber (Abbess Instruments and Systems, Ashland, MA). HR, RR, mean arterial pressure (MAP), and SpO₂ were monitored continuously throughout simulated AE and during the post flight period. Swine were maintained at SpO₂ of 82-85% for hypoxia consistent with 8,000 ft altitude⁵². Arterial blood samples were used to monitor partial pressure of oxygen (PaO₂), potential of hydrogen (pH), lactate, potassium, and hemoglobin at baseline, time of TBI, and then hourly during the 4h simulated flight and during the 4h observation period. Non-survival swine were euthanized following the 4h observation period; survival swine were observed for 7 days post-flight prior to undergoing euthanasia.

Survival Observation

Swine were initially survived and monitored for 7 days post-TBI. Neurological severity score (NSS) was recorded daily on a 32-point scale with 0 being no neurologic deficit and 32 being severe neurologic deficit. The NSS was developed to assess neurological injury via level of consciousness, behavior, appetite, standing position, head position, utterance, gait, and motor function of all four limbs⁵³⁻⁵⁵. Three animals did not recover from the acute TBI and required euthanasia within 24hs of the TBI. Nine animals were survived to POD 7, including five animals in the NS group, two animals in the propranolol group, and two animals in the allopurinol group. The study was then transitioned to a non-survival model due to COVID-induced staffing restrictions on personnel who were needed to monitor the animals. TXA was then added as an additional experimental group for the non-survival analysis, so there were no TBI/TXA animals for 7-day analysis.

Serum Analysis

Serum analysis was completed for non-survival animals only. Arterial blood was collected baseline, time of TBI, and then hourly during the 4h simulated flight and during the 4h observation period. Whole blood was placed in serum separator tubes (BD Bioscience, San Diego, CA) and centrifuged at 1000g for 10 min. Serum was collected and subsequently analyzed for pro-inflammatory

cytokines interleukin-1 β (IL-1 β), IL-6, IL-8, TNF- α using a Qplex Porcine Chemokine High Sensitivity enzyme linked immunosorbent assay according to manufacturer protocol (Quansys Bioscience, Logan, UT).

Immunohistochemistry Analysis

Brain specimens were extracted and preserved following pig euthanasia. The whole brain was placed in a pig brain matrix (Zivic Instruments, Pittsburgh, PA) and 1 cm was removed from the anterior and posterior portions of the specimen. The remaining tissue was separated coronally into two sections, each 2 cm thick. The anterior section contained the area of injury, and the posterior section contained the intact hippocampus. The posterior section was then split into left and right halves at the midline and both sides were fixed separately in 4% paraformaldehyde for 5 days. The tissue was then cryoprotected in 10% glycerol overnight followed by 20% glycerol for 48-72 hs. The specimen was then embedded in a gelatin matrix and returned to the cryoprotectant for an additional 4 days before being frozen in 2-methylbutane at -70 °C. Gross specimens were then stored at -80 °C until sectioning. Tissue was sectioned at 20 micrometre (μ m) on a freezing stage microtome. Free-floating sections were mounted on gelatin-coated slides and allowed to air dry.

Mounted sections were rehydrated prior to heating in 37°C phosphate-buffered saline (PBS) for 10 min. Samples were then incubated with citrate buffer for antigen retrieval (Thermo Fischer Scientific, Waltham MA). Samples were then placed in Bloxall solution (Vector Laboratories, Burlingame, CA) and an Avidin/Biotin block (Biocare Medical, Pacheco, CA) for 10 min each prior to undergoing serum block for 90 min at room temperature with 10% goat serum, 5% bovine serum albumin solution (BSA) and 0.1% triton-X in tris-buffered saline. Slides were then stained with primary antibodies against p-tau recombinant anti-tau phosphor T231 antibody (diluted 1:1000, ab151559; Abcam, Cambridge, UK) overnight at 4°C. A secondary anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was then applied at 1:1000 dilution in PBS-tween for 30 min. Slides then were placed in Vector HRP (Vector Laboratories, Burlingame, CA) and Quanto DAB (Thermo Fischer Scientific, Waltham MA) for 30 min

each prior to counterstaining with hematoxylin and dehydration. Images were captured on Zeiss Axio Imager.M2 microscope (Nikon Corporation, Tokyo, Japan) using the 40x objective, 5ms exposure and 70% intensity. P-tau was quantified using the cell counter feature on ImageJ software by a blinded team member.

Statistical Analysis

All statistical analyses were performed with Prism 6 (GraphPad Software, La Jolla, California). Data are presented as the mean $\pm$ standard error of mean. Two-way ANOVA was utilized to determine whether differences observed were significant. A p value less than 0.05 was considered significant.

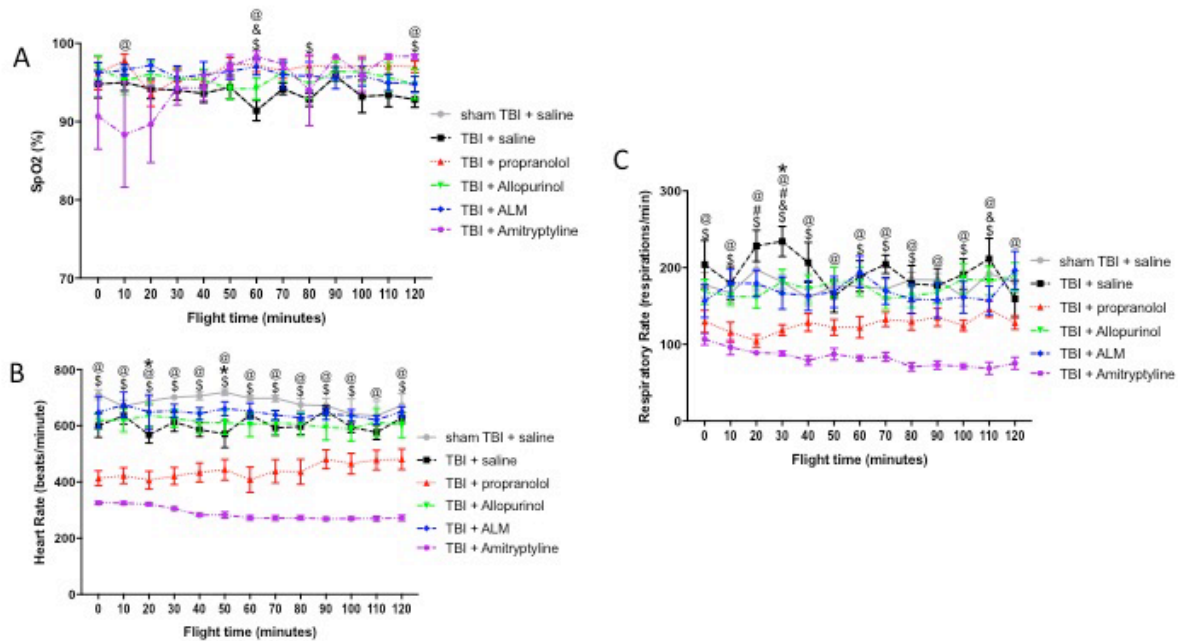
3.0 RESULTS

Murine

Inflight vital signs

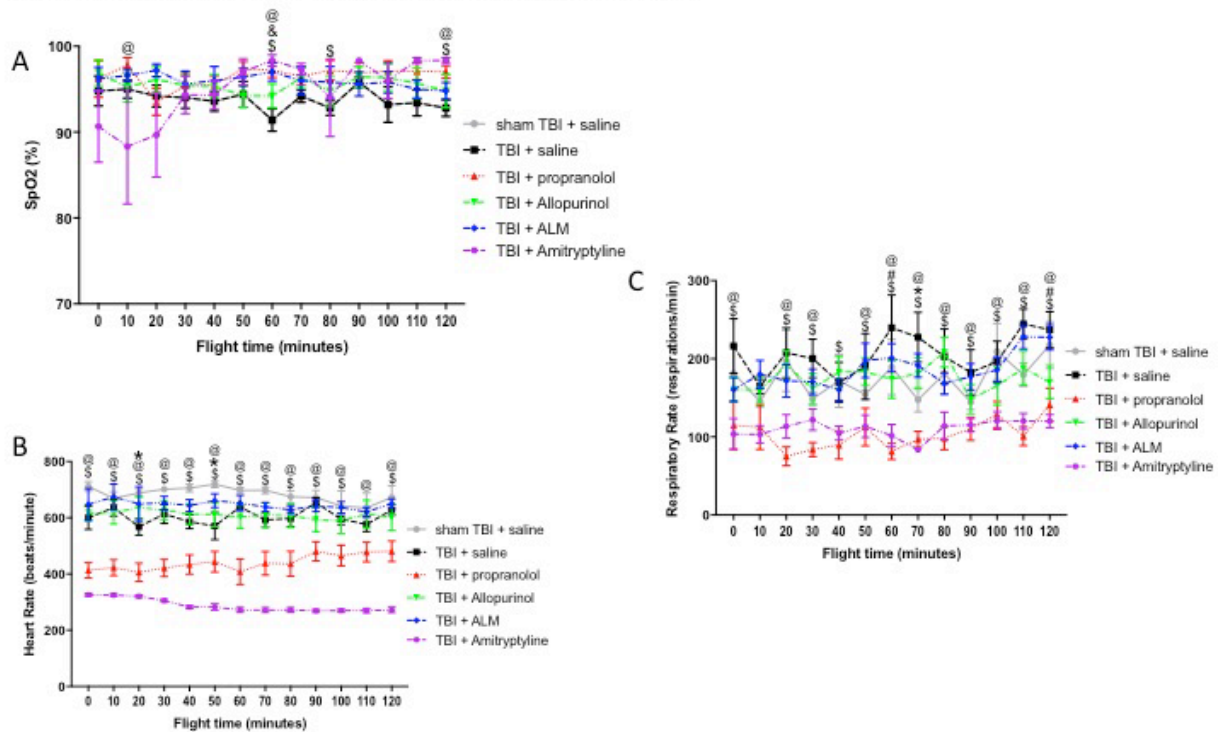
Under NN conditions, TBI/saline mice were found to have no differences in their SpO₂ compared to sham TBI/saline mice, however, differences in HR was noted at 20 min and 50 min into flight (Figure 1a and Figure 1b). RR was different only at 30 min (Figure 1c). In comparison to TBI/saline, TBI/propranolol mice had a higher SpO₂ at 60 min and 120 min into NN exposure while maintain a consistent relative bradycardia and bradypnea. Mice treated with ALM had higher SpO₂ only at 60 min into control flight time. These mice also had a couple of brief time points – at 30 min into flight and 110 min into flight – of relative bradypnea compared to TBI/saline mice. Mice treated with allopurinol displayed only relative bradypnea at 30 min and 110 min. Mice treated with amitriptyline had a lower SpO₂ compared to saline-treated mice at 10 min into flight but subsequently had a higher SpO₂ by 60 min and again the end of the control flight time. These mice also persistently demonstrated bradycardia and bradypnea throughout flight, even more so than the mice treated with propranolol. Of note, mice treated with amitriptyline had a significant mortality rate of 35% after drug treatment.

Figure 1: Vital signs under normobaric normoxic conditions



Under HHH conditions, TBI/saline mice were relatively bradycardic compared to sham TBI/saline mice at 20 min and were relatively tachypneic at 70 min (Figure 2b and 2c). Compared to TBI/saline mice, mice treated with propranolol had improved SpO₂ during HHH while maintaining a relative bradycardia and bradypnea (Figure 2a). Mice treated with ALM also had improved SpO₂ during simulated AE with minimal changes to other vitals throughout flight. Mice treated with allopurinol also had improved SpO₂, though not consistently towards the end of simulated flight. Mice treated with amitriptyline had higher SpO₂ compared to saline treated mice starting at 40 min into flight and persisting until the end of flight. However, they sustained a relative bradycardia and bradypnea.

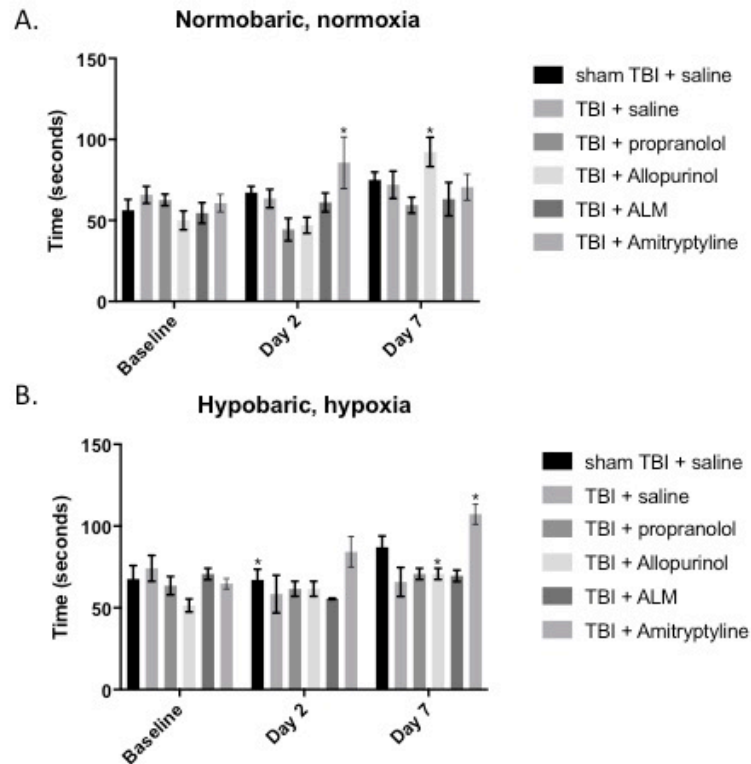
Figure 2: Vital signs under hypobaric hypoxic conditions



Rotarod

Following NN and HHH conditions, neither TBI/saline nor sham TBI/saline showed improvements in their respective baseline times over the course of 7 days. TBI/allopurinol mice were the only group to show improvement on Rotarod performance. These mice also showed improvement under HHH conditions, as did mice treated with amitriptyline. Mice treated with ALM showed some improvement on Day 2 performance, but this was not sustained on Day 7 (Figure 3).

Figure 3: Rotarod performance

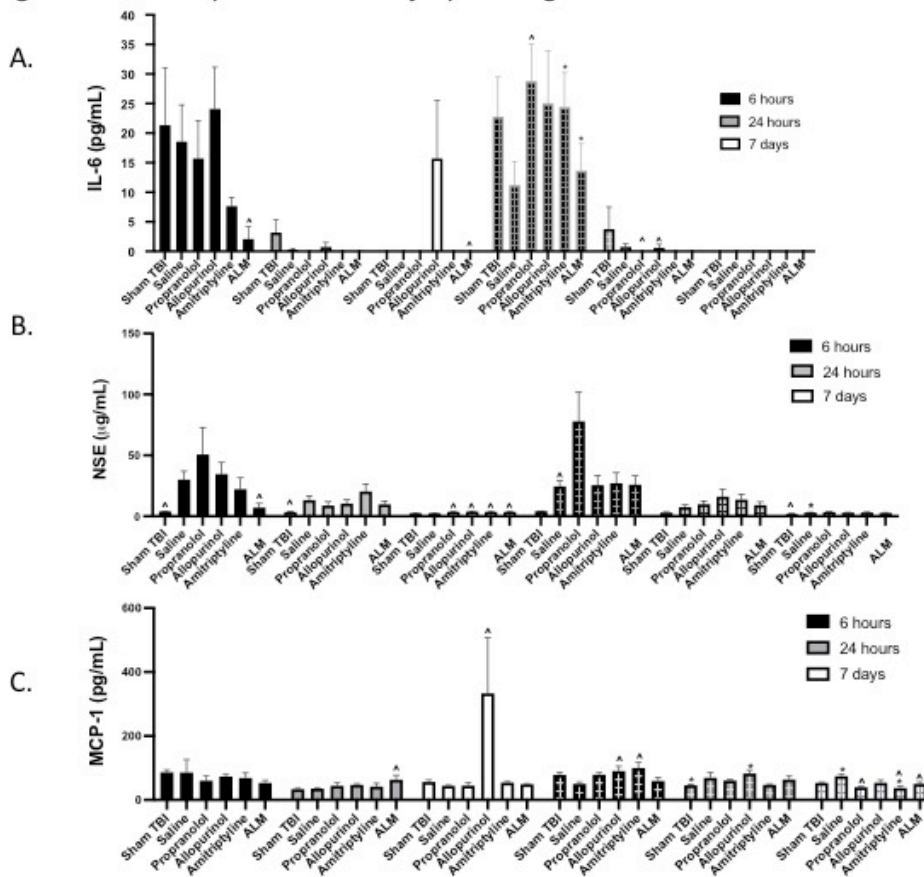


Serum cytokine analysis

Under NN, TBI/saline mice were found to have a higher level of NSE at 6 h but a lower level at 24hr (Figure 4B). TBI/propranolol mice were noted to have increased amounts of NSE at 24 h compared to TBI/saline. TBI/allopurinol mice had elevated NSE as well as MCP-1 at 7 days (Figure 4C). These mice also had a decreased level of serum RANTES at 7 days (supplemental data). TBI/amitriptyline mice showed elevated NSE at 7 days as well as increased RANTES at 6 h, which then decreased at 24 h and 7 days compared to TBI/saline (supplemental data). Mice treated with ALM showed lower serum IL-6 at 6 h and 7 days (Figure 4A). TBI/ALM mice also had reduced NSE at 6 h but an increase at 7 days (Figure 4B). ALM also induced a decrease in TNF α at 24 h (supplemental data), but an elevated MCP-1 at 24 h (Figure 4C). Under HHH, TBI/saline mice had an elevated NSE at 6 h and 7 days compared to sham/TBI saline (Figure 4B). TBI/propranolol mice had elevated

levels of serum IL-6 at 6 h but lower at 24 h compared to TBI/saline (Figure 4A) as well as lower TNF α levels at 24 h (supplemental data). Mice treated with allopurinol had lower levels of serum IL-6 at 24 h and increased serum MCP-1 at 6h compared to TBI/saline (Figure 4A and 4C). Mice treated with amitriptyline had a higher MCP-1 at 6h but this was reduced at 7 days compared to TBI/saline (Figure 4C). Mice treated with ALM only showed a decrease in MCP-1 at 7 days compared to TBI/saline (Figure 4C).

Figure 4: Serum cytokines after injury and flight



Brain cytokines

Under NN conditions, TBI/saline had higher IL-2, IL-3, IL-10, IL-12, IL-17, MIP-1 α , GM-CSF, and RANTES at 6h (supplemental data). By contrast, sham TBI/saline had higher levels of brain IL-1 α , IL-6, and TNF α at 6h compared to TBI/saline (Figure 5A, 5B, 5D). TBI/propranolol mice had significantly less cerebral IL-6, IL-1 β , and IL-3 compared to TBI/saline at 6h (Figure 5B and supplemental data). TBI/allopurinol mice had higher IL-1 β , IL-2, IL-4, IL-10, IL-12, MIP-1, GM-CSF, and RANTES, at 24h compared to TBI/saline (supplemental data). TBI/amitriptyline mice had higher IL-1 α and TNF α at 24 h compared to TBI/saline (Figure 5D). These mice also had higher IL-1 β , IL-2, IL-4, IL-10, IL-12, IL-17, MIP1 α , GM-CSF, and RANTES at 24 h (supplemental data). Finally, TBI/ALM mice showed significantly less cerebral MCP-1 at 6 h compared to TBI/saline (Figure 5C). These mice also had elevated IL-1 β , MIP1 α , and RANTES at 24 h and IL-17 at 7 days compared to TBI/saline (supplemental data).

Under HHH conditions, TBI/saline mice showed decreased cerebral IL6, MCP-1, and TNF α at 6 h compared to sham TBI/saline(Figure 5B-D). TBI/saline mice also had a lower expression of IL-3, IL-4, and RANTES at 6h compared to sham TBI/saline(supplemental data). TBI/propranolol mice had decreased levels of TNF α at 6h compared to TBI/saline (Figure 5D). Mice treated with propranolol also had lower cerebral IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-12, IL-17 at 6h (Figure 5A and supplemental data), as well as higher IL-17, RANTES, and MIP1 α at 24 h (supplemental data). They also had lower MIP1 α and GM-CSF at 7 days (supplemental data). TBI/allopurinol showed decreased IL-1 α , IL-6, TNF α , and MCP-1 at 6h (Figure 5A-D) and increased IL-1 α , IL-6, TNF α , and MCP-1 at 24h compared to TBI/saline (Figure 5A-B and supplemental data). These mice also had a lower IL-10, MIP-1, GM-CSF, RANTES, and IL-17 at 6h but higher IL-1 β , IL-2, IL-3, IL-4, IL-10, IL-12, IL-17, GM-CSF, RANTES, and MIP1 α at 24h compared to TBI/saline (supplemental data). TBI/amitriptyline mice had increased brain IL-1 α , IL-6, and TNF α at 24h compared to TBI/saline (Figure 5A-B and D). These mice also had higher IL-1 β at 24h (supplemental data). Finally, TBI/ALM mice showed significantly lower cerebral IL-6 at 6h and significantly more IL-6 and IL-1 α at 24h compared to TBI/saline (Figure 5A and

B). These mice also had lower RANTES and GM-CSF at 6 h with higher IL-2, IL-3, and IL-10 at 7 days (supplemental table).

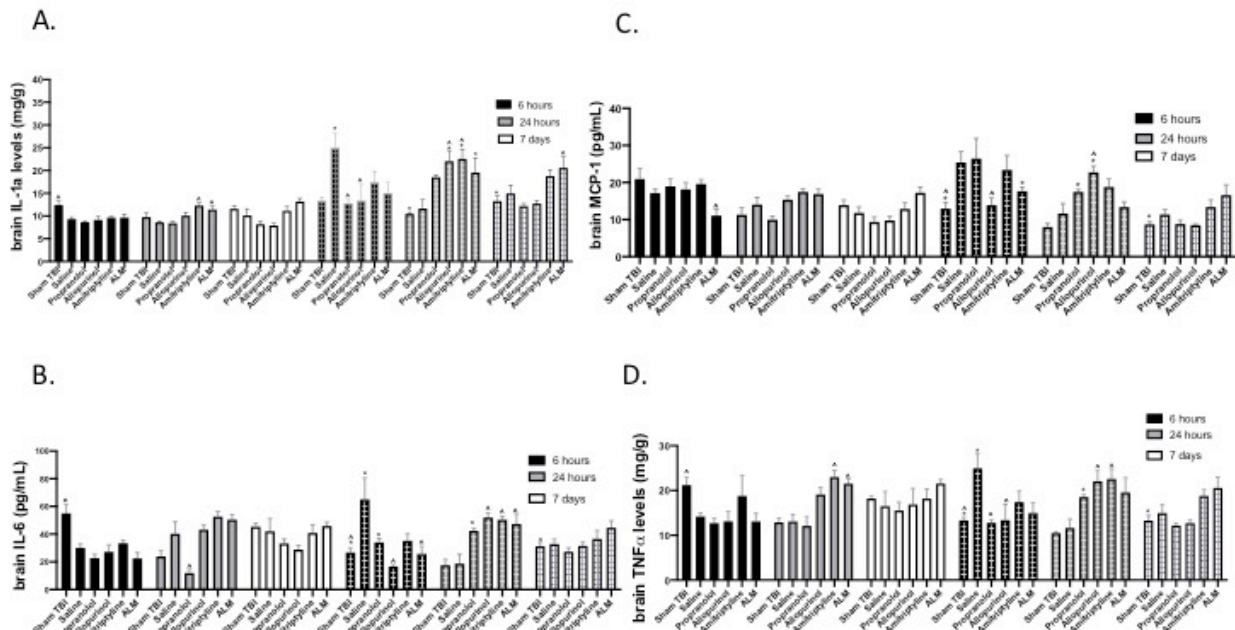
Supplemental Table: Additional cytokine profiles under normobaric normoxic (NN) and hypobaric hypoxic (HH) conditions in serum and brain

		Sham / saline NN	TBI / saline NN	TBI / propranolol NN	TBI / allopurinol NN	TBI / amitriptyline NN	TBI / ALM NN	Sham / saline HH	TBI / saline HH	TBI / propranolol HH	TBI / allopurinol HH	TBI / amitriptyline HH	TBI / ALM HH
TNF- α (pg/ml)	6 hours	12.0	105.8	22.9	10.8	13.0	12.7	13.5	11.7	13.6	12.0	12.3	13.7
	24 hours	11.9	15.4	13.4	10.3*	13.0	10.2*	12.7	16.0	10.9*	13.2	10.9*	12.2
	7 days	12.1	11.2	12.5	12.2	10.2	***	10.2	14.4	10.8	11.1	10.9	***
RANTES (pg/ml)	6 hours	49.6	51.7	42.6	61.5	81.0*	51.0	45.9	48.7	49.6	54.0	57.3	52.0
	24 hours	50.8	72.9	70.8	62.7	43.6*	63.7	58.4	63.0	49.4	72.0	54.9	54.7
	7 days	50.7	66.2	41.7*	48.4*	41.7*	77.2	59.3	61.5	65.9	78.1	67.2	68.8
brain IL-1 β (mg/g)	6 hours	24.4	16.3	15.0*	16.6	18.5	17.3	17.7	25.6	16.6*	15.4*	20.6	19.4
	24 hours	17.7	16.1	16.4	24.7*	32.8*	29.0*	14.5	19.7	26.7	36.1*	33.8*	28.0
	7 days	27.9	22.9	19.3	18.4	27.5	30.2	19.5	22.8	17.5	18.4	22.7	27.2
brain IL-2 (mg/g)	6 hours	31.5*	18.0	14.9	15.0	21.8	21.4	16.7	27.2	11.0*	14.3*	24.7	19.7
	24 hours	16.0	15.2	13.9	25.3*	28.6*	20.6	12.7	18.1	23.4	31.2*	28.0	19.9
	7 days	24.7	17.6	14.3	15.6	20.7	32.6*	14.0	16.8	12.9	11.8	21.2	28.7
brain IL-3 (mg/g)	6 hours	12.8*	8.8	8.0	8.7	9.9	9.1	8.6*	12.4	8.6*	7.4*	10.1	9.6
	24 hours	8.7	10.7	7.8*	12.7	12.7	10.4	7.3	9.8	11.8	15.5	12.9	10.3
brain IL-4 (mg/g)	7 days	11.6	8.9	7.3	8.0	9.5	13.6*	6.8	9.1	7.9	7.8	10.0	13.0*
	6 hours	19.4	12.7	11.3	13.6	12.4	11.0	10.6*	17.2	9.6*	11.8	15.4	13.3
	24 hours	10.2	8.9	9.8	17.1*	23.7*	17.9*	8.2	13.3	19.7	26.1*	22.5	15.5
	7 days	20.1	14.1	11.3	11.6	17.1	23.1*	10.2	14.2	10.9	9.2	15.7	20.4
brain IL-10 (mg/g)	6 hours	19.9*	8.8	8.0	13.8	9.3	10.1	11.8	22.1	10.7	9.5*	11.6	18.2
	24 hours	8.7	8.6	8.8	16.3*	17.4*	14.4	9.9	11.9	14.0	20.0*	13.3	11.3
	7 days	15.5	15.0	9.3	15.5	21.2	16.1	11.2	12.1	7.4	14.1	12.5	20.1*
brain IL-12 (mg/g)	6 hours	55.4*	33.5	28.7	33.1	34.2	28.1	31.9	49.1	23.4*	27.7	40.7	36.6
	24 hours	30.9	34.2	26.6	51.4*	62.0*	43.6	29.0	36.4	57.3	76.8*	63.0	45.3
	7 days	55.1	34.9	33.8	32.1	48.4	58.9	32.5	40.6	33.2	36.9	46.6	59.8

brain IL-17 (mg/g)	6 hours	18.6*	14.0	14.2	15.6	16.3	16.1	16.7	23.1	15.7*	14.6*	18.6	16.6
	24 hours	14.9	15.2	15.5	17.6	21.4*	16.4	14.8	16.6	19.9*	25.1*	19.1	17.1
	7 days	16.8	15.9	13.3*	15.5	17.1	23.2*	12.5	14.8	13.6	15.4	19.0	21.9
brain MIP-1 α (mg/g)	6 hours	22.3*	11.3	11.3	13.9	11.0	13.8	13.2	21.2	12.7	9.5*	18.9	15.6
	24 hours	12.6	8.6	13.8	19.6*	21.3*	19.0*	13.3	13.8	22.1*	30.2*	22.9	19.1
	7 days	18.9	13.1	17.0	12.3	20.3	21.5	15.2	15.4	10.6*	14.0	21.3	24.2
brain GM-CSF	6 hours	12.7*	5.7	7.1	7.3	4.8	4.6	5.6	13.1	5.6*	4.2*	8.9	5.4*
	24	5.4	4.9	4.0	10.8*	10.6*	8.1	4.5	6.9	10.7	15.4*	9.1	8.7

(mg/g)	hours												
	7 days	8.0	4.5	4.6	4.0	7.4	11.4*	4.6	6.0	3.6*	4.3	6.6	10.8
brain RANTES	6 hours	18.2*	12.1	11.3	13.3	12.9	10.1	8.6*	19.9	11.5	9.9*	14.2	10.6*
	24 hours	10.4	10.7	8.7	18.7*	23.0*	19.8*	8.1	11.1	23.3*	28.6*	22.6	17.8
	7 days	18.1	14.3	11.9	12.3	20.0	20.0	13.0	14.7	12.7	13.7	18.1	20.0

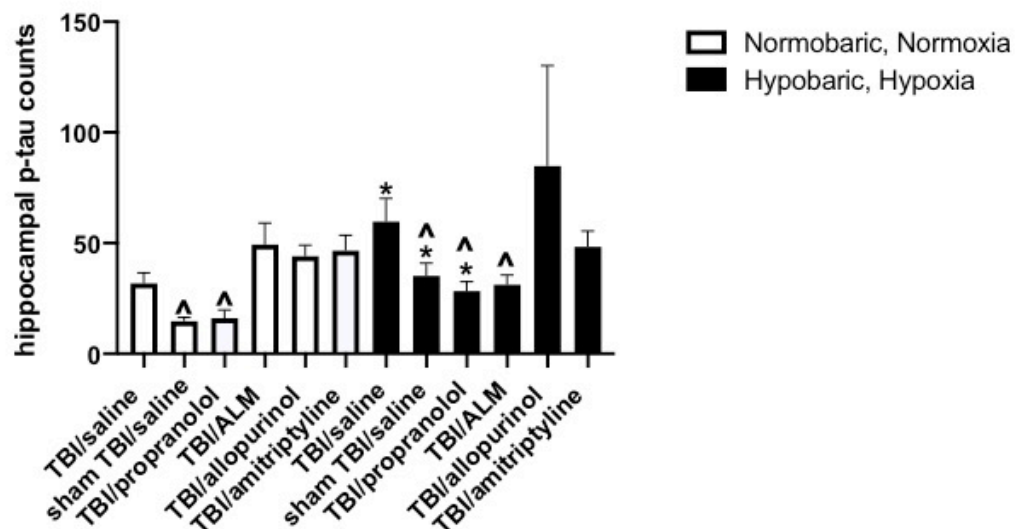
Figure 5: Brain cytokines after injury and flight



30 Day p-tau accumulation

At both NN and HHH, TBI/saline mice had significantly more p-tau accumulation compared to sham TBI/saline mice (Figure 6). Interestingly, both TBI/saline mice and sham TBI/saline mice had an increase in p-tau accumulation in the setting of HHH compared to NN. Similarly, TBI/propranolol mice had a more p-tau accumulation under HHH conditions compared to NN but less than what was observed in TBI/saline under both conditions. TBI/ALM mice did not show differences in p-tau accumulation compared to TBI/saline under NN but did have reduced hippocampal p-tau compared to TBI/saline under HHH. No differences were seen in TBI/allopurinol or TBI/amitriptyline mice compared to TBI/saline or in comparing mice treated with these drugs subjected to both NN with HHH conditions.

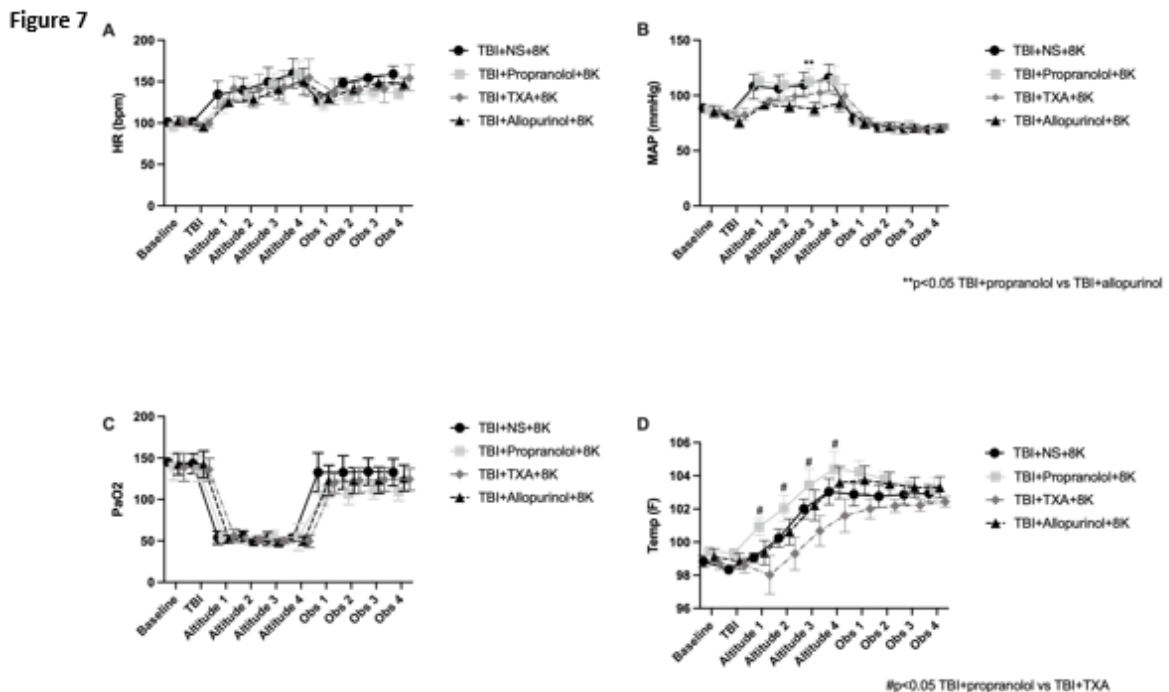
Figure 6: 30-day p-tau accumulation



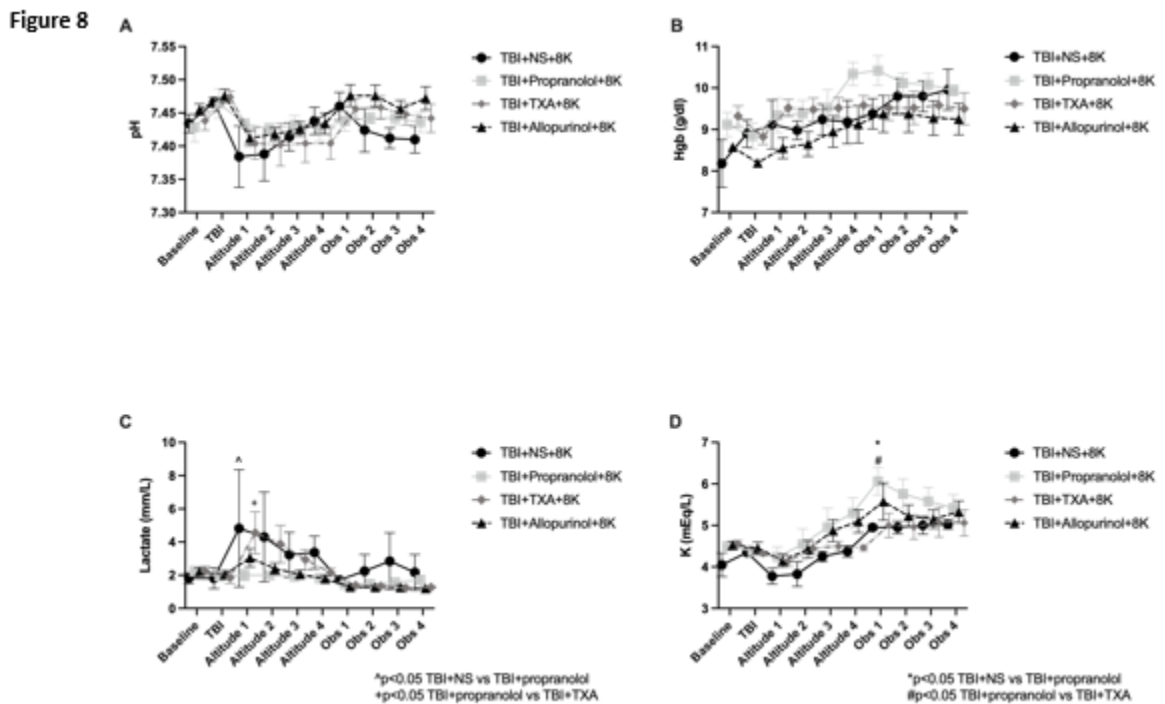
Porcine

Hemodynamics

There were no significant differences in HR, MAP, PaO₂, or temperature between non-survival treatment groups and saline control during experimentation (Figure 7 A-D). Importantly, this included a lack of hemodynamic effect from the propranolol administration. The model of hypobaric hypoxia was confirmed with significant decreases noted in PaO₂ during altitude with significant improvements during observation within all groups. There was a transient significant difference in MAP between TBI+propranolol and TBI+allopurinol during h three of flight that otherwise resolved for the rest of experimentation, with the propranolol group having the higher MAP ($p < 0.05$, **Figure 7B**). Temperature was significantly higher in the TBI+propranolol group compared to the TBI+TXA group during 4 h of flight, however, was unchanged during observation ($p < 0.05$, **Figure 7D**). Cerebral blood flow and tissue oxygenation remained unchanged between treatment and control groups during and after simulated flight.



Blood analysis revealed no significant differences in pH or Hemoglobin (Hgb) between treatment groups and saline during or after flight (**Figure 8A and B**). Serum lactate was noted to be significantly decreased 1 h after flight in the TBI+propranolol group compared to the TBI+NS group but this difference resolved after this time point ($p<0.05$, **Figure 8C**). Potassium was significantly elevated in the TBI+propranolol group compared to both TBI+NS and TBI+TXA groups during the first h of observation however this difference was not sustained past this time point and did not have any other effects such as arrhythmia ($p<0.05$, **Figure 8D**).

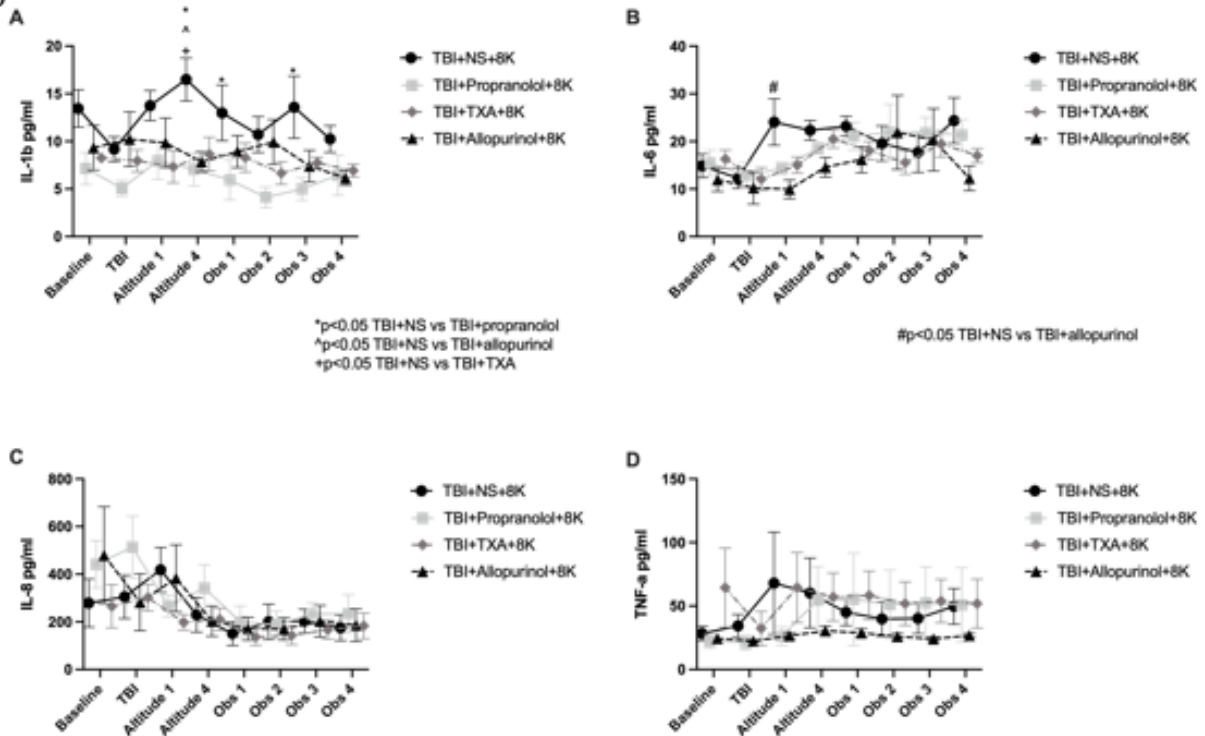


Serum Cytokines

Serum IL-1 β was significantly decreased in TBI+propranolol group compared to TBI+NS from h 4 of flight to h 3 of observation ($p<0.05$, **Figure 9A**). Similarly, IL-1 β was decreased in TBI+allopurinol and TBI+TXA compared to TBI+ NS at h 4 of flight but this did not persist after flight ($p<0.05$, **Figure 9A**). IL-6 was significantly decreased in TBI+allopurinol at h 1 of flight compared to

TBI+ NS ($p < 0.05$, **Figure 9B**). IL-8 and TNF- α were not significantly different between groups during or after flight.

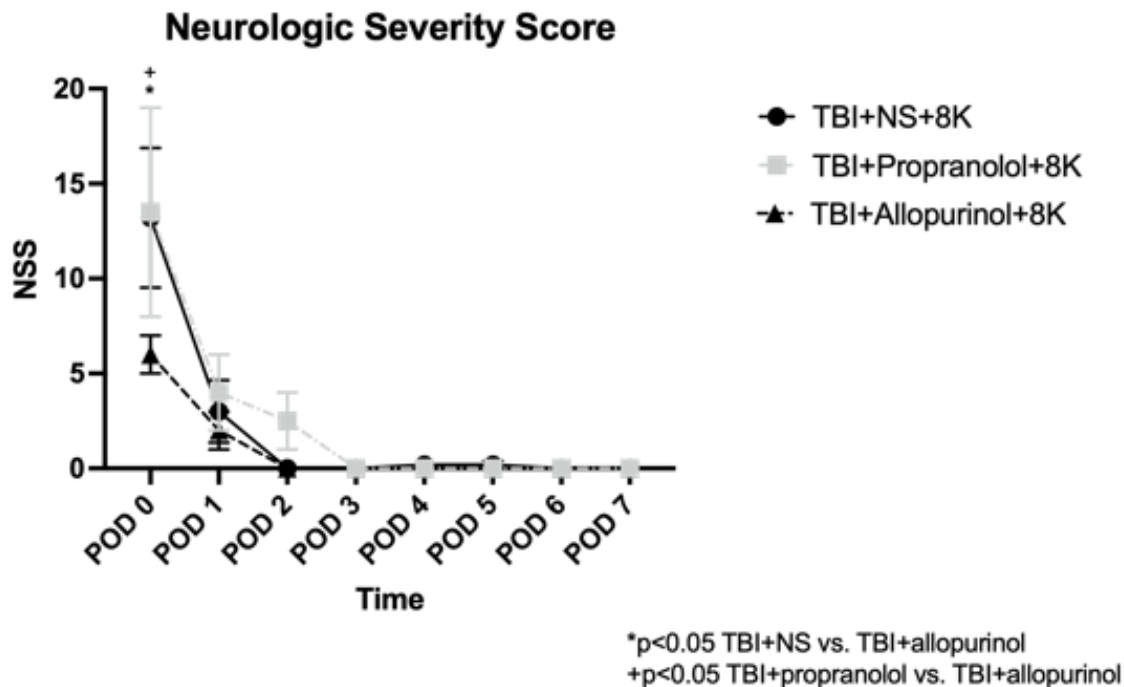
Figure 9



Neurologic Recovery

NSS was recorded at 4–6 h after the 4-h observation period, and then daily until post injury day seven in all 7-day survival cohorts. Pigs in the TBI+allopurinol group had significantly lower NSS (6.0 ± 1.0) compared to TBI+NS (13.2 ± 3.7) and TBI+propranolol (13.5 ± 5.5) on the day of injury ($p < 0.05$, **Figure 10**). This effect did not persist past 24 h post-TBI. Other than the three animals which did not recover, all other survival pigs recovered to NSS of zero by post-operative day 3 of experimentation.

Figure 10

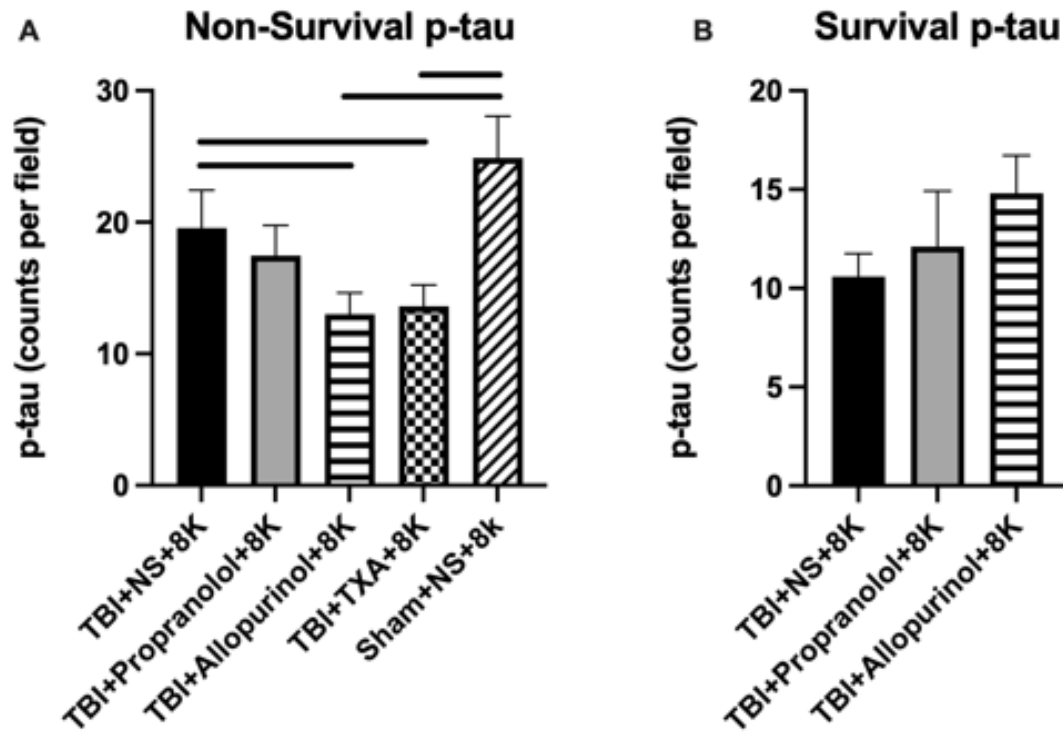


Immunohistochemistry

Non-survival pigs were euthanized after four h of observation following flight.

Immunohistochemistry of bilateral hippocampal regions from non-survival specimens revealed a significant decrease in p-tau accumulation in animals treated with allopurinol (13.00 ± 1.64) and TXA (13.60 ± 1.63) compared to the NS group (19.55 ± 2.88) with flight ($p < 0.05$, **Figure 11A**). Significant differences were also noted in the non-survival animals treated with allopurinol (13.00 ± 1.64) or TXA (13.60 ± 1.63) compared to the sham TBI group (24.90 ± 3.16) ($p < 0.05$, **Figure 11A**), demonstrating a possible effect of altitude alone without TBI. There were no significant differences in p-tau accumulation at 7 days for the survival animals treated with either NS, propranolol, or allopurinol (**Figure 11B**).

Figure 11



4.0 DISCUSSION

Murine

We compared allopurinol, propranolol, ALM, and amitriptyline to saline in the prevention of secondary brain injury from HHH conditions following TBI. These drugs demonstrated variable outcomes.

Propranolol maintained lower heart and respiratory rates with some reduced pro-inflammatory properties but without functional improvements. Amitriptyline also induced lower post-TBI respiratory and heart rates and improved motor function outcomes without an effect on the systemic inflammatory response. While allopurinol did not affect respiratory and heart rate, it improved late post-TBI systemic inflammation as well as functional outcomes. By contrast, ALM provided no consistent improvement in physiologic, inflammatory, or functional outcomes but did reduce p-tau accumulation under HHH.

Taken together, allopurinol had the most favorable profile in examining short term outcomes after TBI and HHH exposure by decreasing inflammatory cytokines and improving motor function, however, propranolol had the most favorable long-term profile with a decrease in hippocampal p-tau accumulation.

The need for effective pharmacotherapies to ameliorate secondary injury after TBI in the military is paramount. Most civilian and military flights occur with a cabin pressurized to 8,000ft – an altitude at which there can be concerns for lower oxygen saturations, even among healthy individuals ⁵⁶. This environment presents a unique challenge to medical management of TBI, as the pharmacotherapy not only has to work in the setting of TBI but also has to protect in the setting of hypobaric hypoxia, and potentially hyperthermia as well. A retrospective study of 63 patients with TBI who underwent rapid evacuation with the Air Force's Critical Care Air Transport Team demonstrated that 53% of patients had at least one secondary cerebral insult ⁵⁷. The most common effect was hyperthermia, however, 25% of patients also had experienced hypoxia ⁵⁷. Another study investigating the effects of aeromedical transport on TBI showed that intracranial pressure (ICP) can vary as much as 50% from baseline, with the most severe changes occurring during takeoff and landing ⁵⁸. At the present time, no

pharmacotherapy has been proven to help ameliorate these outcomes, although some have suggested that adjusting sedation can help with ICP variation⁵⁸.

Propranolol has been suggested by multiple murine studies and has some confirmation in human studies to be beneficial after TBI^{17,18,20,59}. However, the mechanism by which propranolol improves outcomes has yet to be elucidated. One hypothesis is that propranolol decreases post-traumatic inflammation. However, a study by Zeeshan et al showed improvement in post-TBI cognition without corresponding improvement in pro-inflammatory markers, such as IL-1 β , IL-6, and TNF α ²⁷. Our TBI/propranolol mice similarly showed no significant changes in these cytokines compared to TBI/saline controls; however, TBI/propranolol mice importantly exhibited less p-tau accumulation at 30 days, suggesting a separate pathway independent of the inflammatory response for preventing the long term hippocampal structural changes. Similarly, existing literature suggests that propranolol may improve cerebral hypoxia in the setting of TBI⁶⁰. However, cerebral hypoxia was assessed under NN conditions as a function of changes in flow. Nevertheless, our studies demonstrated that mice treated with propranolol had some level of resiliency for cerebral hypoxia compared to TBI mice treated with saline. While overall HHH conditions led to worsening injury than observed in NN, TBI/propranolol mice had improved vital signs, some biomarkers of inflammation, as well as decreased p-tau at 30 days compared to saline controls.

We also found that ALM had decreased p-tau accumulation at 30 days compared to saline. However, this was only true under HHH conditions and no changes were seen with NN. We also did not find the improvements in the inflammatory cytokine profile that Letson et al demonstrated in their rat model⁴⁰. However, we only gave a single dose of ALM, whereas their group gave a bolus intravenously followed by an infusion, suggesting that a higher or sustained dose of ALM may require for an improvement in the inflammatory cytokine profile.

Interestingly, one hypothesis of why allopurinol is protective in TBI is the increased generation of ATP and adenosine⁶¹. In our experiment, mice treated with allopurinol had the most favorable short-term outcome profile and was the only cohort with improvement in Rotarod performance. They also

showed an improved cytokine profile, with higher SpO₂ during HHH. Perhaps the dosage of allopurinol administered produced a greater amount of adenosine than what the ALM mice were treated with directly, however, we did not measure this. Alternatively, xanthine oxidase is thought to be one of the major sources of superoxide radicals after ischemia reperfusion and is perhaps responsible for some of the subsequent injury observed⁶². While we did not measure xanthine oxidase levels explicitly, the decreased pro-inflammatory profile could reflect this change. Allopurinol is currently being tested in a phase III human trial for improving outcomes after perinatal asphyxia⁶³. Interestingly, this administration is in conjunction with a hypothermic protocol, which may have variable results in the setting of TBI, as cooling after head injury has been unsuccessful in several clinical trial⁶⁴⁻⁶⁶.

TBI/amitriptyline treated mice had a partially favorable pro-inflammatory profile, however, the bradycardia and bradypnea during flight were beyond protective, and indeed, potentially harmful, as approximately 35% of the mice died between injection and the end of flight. Perhaps more interesting was the lack of functional improvements observed in these mice, as amitriptyline is considered to convey functional benefits after TBI, particularly with respect to agitation and headache^{36,62}. Our lab group has previously demonstrated improvements in depression after TBI in a murine model³⁹. However, this may not translate to improvements in motor functionality, which is closer to what Rotarod tests. Additional post-TBI depression testing was not done in the current study and may be the foundation of future studies to understand the implications of HHH after TBI.

This phase of our study has several limitations. First, this is a rodent model of TBI and there are significant differences between what may be observed in a mouse compared to a human after TBI. Similarly, there are notable cerebral morphological differences between the two species that may contribute to varied responses to both injury and treatment. Our model of TBI is strictly blunt force and lacks the rotational and angular forces that are typically present in battlefield patterns. It is also a model of moderate TBI and so our findings here may not translate to lesser or more severe injuries. In terms of the drugs investigated, both propranolol and amitriptyline are sodium channel antagonists, which can be associated with an increase in seizures. While propranolol is associated with seizures in the setting of

overdose, this direct adverse effect has not been reported in the setting of TBI⁶³. Amitriptyline has been associated with lowering seizure threshold in those who have a history of seizures and should be used with caution in the setting of TBI⁶⁹. Our results also indicate that the neurologic and inflammatory benefits to administering amitriptyline may be limited. Finally, our studies demonstrated a variety of ways in which pharmacotherapy can influence outcomes after TBI and HHH exposure – namely oxygenation, inflammatory response modulation, functional outcomes, and long-term structural changes such as p-tau accumulation. None of the drugs tested showed a singular dominance in all domains, nor was that an expected outcome. However, our study is able to demonstrate which of the drugs shows the more favorable profile for each of the potentially desired outcomes.

Porcine Phase

This study aimed to determine if clinically utilized medications including allopurinol, propranolol, or TXA would mitigate the secondary insult experienced during AE following TBI. There were no significant differences in HR, MAP, PaO₂ or temperature between treatment groups and saline controls. Intermittent differences in physiologic markers and serum cytokines were observed but did not persist through the entire observation period. Importantly, there were no adverse effects to hemodynamics or inflammation after administration of propranolol, allopurinol or TXA. P-tau accumulation was decreased post-TBI in the non-survival allopurinol and TXA groups but was unchanged on post-injury day 7 in all survival groups. Overall, these data demonstrate that these medications are safe to administer in TBI patients prior to AE and may improve p-tau accumulation within the hippocampus.

Propranolol, a nonselective lipophilic beta-adrenergic receptor blocker, has been shown in randomized control trials to decrease cerebral inflammation following TBI^{17,18,25,27}. It has also been shown in murine models to decrease p-tau and amyloid beta plaque accumulation within the hippocampus²⁶. Although a clinical benefit has been observed in humans and animal models alike, the specific mechanism of action remains unknown. To our knowledge, the administration of propranolol in TBI patients prior to AE has not been studied. Although there are clinical concerns about the

hemodynamic effects of early post-injury beta-blocker administration, our study demonstrated no change in hemodynamics during or after flight with administration of propranolol. In fact, lactate was significantly decreased during the first h of flight within the propranolol group compared to pigs treated with NS. Propranolol treatment led to decreased IL-1 β during the end of flight and during the first three h of observation. Zeeshan et. al. similarly found no significant differences in IL-1 β , IL-6 and TNF- α on day 7 following TBI using a murine model²⁷. NSS was unchanged in pigs treated with propranolol compared to normal saline. This was also demonstrated in a study by Zlotnik et. al. which found no significant difference in short term functional outcome after administration of propranolol in a murine model⁷⁰. With no observed benefit in systemic inflammation or short-term functional status, the clinical improvement noted in various human studies could be related to attenuation of p-tau formation within the hippocampus. Our previous murine model demonstrated decreased p-tau accumulation at 30-days in mice treated with propranolol post-TBI⁵. This study failed to demonstrate a significant difference in p-tau accumulation within the hippocampus at 7-days post injury, which may be due to shorter time from injury to analysis in a larger animal model. Further studies will be necessary to elicit the optimal duration after injury and mechanism by which propranolol inhibits p-tau accumulation within the hippocampus

Allopurinol, a xanthine oxidase enzyme inhibitor, has previously been shown to have neuroprotective effects in 7-day old rats after hypoxic ischemia²⁹. Other murine studies have demonstrated improvement in short-term and long-term functional outcomes when administering allopurinol following hypoxic/ischemic insult^{71,72}. This benefit did not translate to humans when administered to babies after experiencing perinatal hypoxic ischemia⁷³. Few studies have evaluated the theoretical benefit of allopurinol following TBI. It is postulated that the antioxidant effects of this medication help to reduce free radicals and attenuate reperfusion injury^{74,75}. Our study demonstrated decreased IL-6 levels in allopurinol treated animals during the first h of flight compared to normal saline treatment. Allopurinol demonstrated no adverse changes in hemodynamics during or after flight, suggesting that it may be safe to use in TBI patients and in the hypobaric hypoxic environment innate to

AE. Our previous murine model demonstrated improved short term neurological outcomes in mice treated with allopurinol via balance-related learning evaluations⁵. This study similarly demonstrated improvement in short term functional status as NSS was noted to be significantly improved in allopurinol treated animals during the seven-day survival period. Further long-term observation will be needed to determine if the functional improvement persists. P-tau accumulation within the hippocampus was attenuated in non-survival animals treated with allopurinol prior to flight. However, this benefit did not persist in the survival animals to post-injury day seven. This also supports the findings that allopurinol is beneficial in the immediate post-TBI period but may not have lasting effects.

Immediately following TBI, cerebral tissue injury is associated with a unique coagulopathy associated with tissue factor release. Intracranial bleeding also contributes to the neuroinflammatory environment within the brain parenchyma. TXA is a synthetic reversible competitive inhibitor of the lysine receptor on plasminogen preventing the formation of plasmin. Plasmin, a component within the fibrinolysis pathway, has been shown to exert inflammatory effects through the activation of complement, monocytes, and neutrophils⁷⁶. The Clinical Randomization of an Antifibrinolytic in Significant intracranial Hemorrhage 3 (CRASH-3) trial demonstrated that TXA was beneficial after isolated TBI as well as polytrauma when administered within 3 h of injury³². Post-hoc analysis of the CRASH-3 data demonstrated that TXA improved 24-h mortality after TBI but showed limited improvement in mortality after 24 h³¹. Our data revealed no significant changes in hemodynamic status or physiologic markers in TXA treated animals compared to NS treated animals. Cytokine analysis revealed a significant decrease in IL-1 β in TXA treated pigs compared to NS controls during the last h of flight only. Previous studies within orthopedic patient populations have demonstrated decreased IL-6 following peri-operative dosing of oral TXA and intraarticular TXA injection with knee replacement⁷⁷. Although small perioperative clinical studies have demonstrated decreased systemic inflammation with TXA administration, previous murine models have failed to demonstrate significant benefit. Our group has previously demonstrated a similar lack of significant changes in systemic inflammatory cytokines with TXA administration following TBI in a murine model⁷⁸. Further research is needed to determine

the interaction between TXA and the immune response following TBI. Immunohistochemistry revealed that TXA may have potential benefit in mitigating p-tau accumulation within the hippocampus. Unfortunately, there were no animals within the survival cohort to receive TXA. Additional research will be necessary to determine if the attenuation of p-tau development persists with TXA administration.

One significant limitation of our study is that the survival portion of the study was terminated prior to completion of all study groups. This was due to COVID restrictions limiting personnel necessary for monitoring animals. We intended to survive each group of five pigs for 7 days post TBI/flight to assess p-tau accumulation within the hippocampal region. Although this portion of the study was terminated early, nine animals were survived to POD 7. These animals were utilized to evaluate NSS and p-tau accumulation within the hippocampus following moderate TBI and AE. No survival animals were treated with TXA, as this treatment arm was added to the non-survival arm of the study after termination of the survival study. Therefore, we are unable to comment on the functional outcomes of this medication or accumulation of p-tau within the hippocampus. When assessing the nine animals that were survived to POD 7, the NSS scores for normal saline were similar to control groups in previous studies further supporting this method of assessing neurological function after TBI⁷⁹. Another limitation of our study was that each pig only received a single dose of the treatment medication following TBI. Prospective clinical studies have shown that repeated doses of propranolol in humans show benefit in short- and long-term neurological outcomes^{18,19}. We administered only one dose of each medication to determine whether a single dose prior to AE could attenuate the secondary injury that is caused by the hypoxic hypobaric environment. More research must be completed to determine optimal dosing of medications and frequency of repeat dosing. Future projects may include incorporating hemorrhage into the injury model, increasing altitude during AE, and extending survival to 60-90 days for temporal analysis of p-tau accumulation within the hippocampus.

5.0 CONCLUSION

Taken together, our study illustrates that there may be three potential medications, allopurinol, propranolol, and TXA that confer lasting neurological benefit by preventing secondary injury after AE. These benefits may be by regulating vital signs or by improving the post-TBI hypoxia-induced inflammatory response, functional outcomes, and long-term neuronal changes. Multimodal pharmacological interventions may be needed to provide comprehensive post-TBI care and protection, as provided during human clinical care. In the future, further investigation will be needed to determine any persistent behavioral or neurological effects of injury, flight, and a combined multimodal pharmacologic treatment.

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ACRONYMS/ABBREVIATIONS & SYMBOLS

%	percent/percentage
±	plus or minus
<	Less-than
°C	degrees of Celsius
cm	centimeter(s)
g	gram(s)
h	hour (s)
kg	kilograms
L/min	liter per minute
mg	milligram(s)
m/kg	meter per kilogram
mg/kg	milligrams per kilogram
mM	Millimolar
mg	milligram(s)
mg/ml	milligram per milliliter
min	minute/minutes
mm	millimeter(s)
mL	milliliter
m/s	meter per second
µm	micrometre
uL/g	microliter per gram
p	power
X	times
AE	aeromedical evacuation
ALM	adenosine/lidocaine/magnesium
BCA	bicinchoninic acid
BSA	Bovine serum albumin solution
CCI	controlled cortical injury
COVID	Coronavirus disease
CRASH-3	Clinical Randomization of an Antifibrinolytic in Significant intracranial Hemorrhage 3
ELISA	enzyme-linked immunoassay
ft	feet
GM-CSF	granulocyte-macrophage colony-stimulated factor
Hgb	Hemoglobin
HHH	hypoxia, hypotension, and hypercarbia
HR	Heart Rate
ICP	intracranial pressure
IL	interleukin
IL-1α	alpha cytokine
IL-1β	Interleukin 1-beta cytokine
IL-2	Interleukin 2 cytokine
IL-3	Interleukin 3 cytokine
IL-4	Interleukin 4 cytokine
IL-6	Interleukin 6 cytokine
IL-10	Interleukin 10 cytokine
IL-12p70	Interleukin 12 cytokine heterodimeric pro-inflammatory cytokine
IL-17	Interleukin 17 cytokine

IL-8	Interleukin 8 cytokine
MAP	mean arterial pressure
MCP-1	monocyte chemoattractant protein-1
MIP-1 α	Macrophage Inflammatory Protein-1 α
n	number of particles in the substance; or number of subjects in a study group
NN	normobaric normoxia
NS	normal saline
NSE	Neuron-specific enolase
NSS	Neurological severity score
phosphorS262	polyclonal antitau antibody
PaO ₂	partial pressure of oxygen
PBS	phosphate-buffered saline
pH	potential of hydrogen
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RR	respiration rate
SpO ₂	oxygen saturations
TBI	Traumatic Brain Injury
TNF α	tumor necrosis factor alpha (multifunctional cytokine)
TXA	tranexamic acid