BEGIN WHOLE ANIMAL RAT EXPERIMENTS

EXPERIMENT SET 1) Determine the appropriate route and dose of MitoQ to administer to rats. OVERVIEW: This experiment has two parts. FIRST, acute toxicity testing is done to establish the safe dose of MitoQ as given by three different parenteral delivery routes: intravenous (i.v.), intranasal (nas.), and intramuscular (i.m.). SECOND, once the safe dose of MitoQ for each route of delivery (i.v., nas., i.m.) is determined, then the levels of MitoQ accumulation in the brain, blood and lungs will be determined as a function of time after dosing: times 0, 0.5, 1, 2 & 4 hr post-injection. Tissues harvested postmortem will be analyzed using liquid chromatography-dual mass spectroscopy. OUR GOAL is to identify the best route and dose that elevates brain levels of MitoQ within <1 to 2 hr.

EXPERIMENT SET 1 IS NECESSARY because to date, MitoQ has been delivered to rats by adding it to their drinking water for 2-8 weeks. When MitoQ was delivered in this manner, it reached steady state levels in the brain and other tissues after 7-10 days. Once stopping treatment, MitoQ was quickly washed out (half-life = 1.5 days). In mice, however, a single i.v. injection (tail vein) of MitoQ in Swiss-Webster mice elevated levels in brain tissue in <1 hr with >90% clearance after 24 hr. Extrapolation of dosages from mice to rats is challenging since mice have a higher metabolism and thus often require a higher dose of test substance relative to that used in rats. This issue, however, will be quickly resolved using the up-and-down acute toxicity method in which the safe dose of substance can usually be achieved testing 6-10 animals (Lipnik R.L. et al. (1995) Comparison of the Up-and-Down, Conventional LD50, and Fixed Dose Acute Toxicity. Procedures. Fd. Chem. Toxic. 33(3): 223-231; and Dixon (1991) Staircase Bioassay: The Up-and-Down Method. Neuroscience & Behavioral Reviews. 15: 47-50). Up-and-Down Acute Toxicity Testing: based on the literature, 6-10 rats/delivery route will be needed to determine the safe vs. toxic dose of MitoQ. Using the highest number, we estimate that 30 rats will be needed to test 3 routes of delivery: [(3 routes: i.v., nas., i.m.) x (1 MitoQ) x (10 rats max. /route)] = 30 rats

Liquid Chromatography Analyses: Once the upper limit for safe dosing with MitoQ is determined, we will then quantify its levels in brain tissue for each delivery route (i.v., nas. & i.m.) using liquid chromatography-dual mass spectroscopy. Animals will be dosed with MitoQ and then blood samples, lung samples, and brain regions (brain stem, hippocampi & cerebral motor cortices) harvested at 0.5, 1, 2 & 4 hr post-injection of MitoQ to measure levels of MitoQ. A fifth group of control animals without MitoQ treatment will be euthanized to collect baseline levels of MitoQ (i.e., time 0 and presumably zero levels of MitoQ) and to assess blood redox status. Animals are anesthetized using 3-5% isoflurane and an arterial blood sample taken (cardiac puncture under isoflurane anesthesia) to measure the rat's redox status using the redoxSYS analysis meter (40uL blood required per sample for analysis). The animal is then euthanized and brain tissues are dissected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Based on a previous study, we plan to use six animals per group: [(6 rats/group) x (3 groups: i.v., nas., i.m.) x (1 substance: MitoQ) x (4 time points: 0.5, 1, 2 & 4 hr post-MitoQ injection)] + (6 rats: baseline group, time 0 (no MitoQ injection)) = 78 SD rats

EXPERIMENT 2) Expose freely behaving rats to HBO2 -/+ MitoQ. OUR GOAL is to determine the effect of MitoQ on the latency time to onset of visible seizures. Unoperated rats (without radio-telemetry modules implanted) will be dosed with MitoQ, TPMP (triphenylphosphonium cation--the molecule that is bonded to coenzyme Q to render MitoQ highly permeable to the cell membrane and mitochondrial membrane) or vehicle (soybean oil) using the protocol that provides the highest level of MitoQ in brain tissues without toxicity (determined in Experiment 1). In parallel with MitoQ/vehicle dosing, each rat is

adapted daily to the hyperbaric chamber, 1 hr/day x 7 days, before being dived on HBO2. After treatment with MitoQ, a single rat is placed in the animal chamber that is housed inside the hyperbaric chamber. The animal chamber is ventilated with 1 atmosphere absolute (ATA) air for at least 30 min before switching to 1 ATA O2 for another 15 min (note: 1ATA pressure = sea level = room pressure). The animal is then dived (~1 atmosphere gauge pressure, ATG/min) to 5 ATA O2. The animal is held at maximum depth until onset of first and second bouts of visible tonic-clonic seizures, which are separated by an interictal period that can range for tens of seconds to tens of minutes. The maximum time exposed to HBO is 2 hours; if seizures have not occurred then the HBO exposure is terminated. Thus, the maximum total time inside the chamber is 2h, 45min. Each animal has access to 5-6 pieces of food kibble lying on the floor of the animal chamber and a Hydrogel packet for drinking. The intensity of each bout of visible seizures is scored using our six-stage scale for CNS-OT seizures. Following the second bout of seizures, the animal is changed over to air and decompressed back to sea level (~1 ATG/min). Blood samples will be taken immediately following the dive (cardiac puncture under anesthesia) to measure the animal's redox status using the redoxSYS analysis meter (40uL/sample). Based on our past studies, we plan to use 10 rats/group: [(10 rats/group) x (3 treatments: MitoQ, TPMP & vehicle) = 30 SD rats.

EXPERIMENT 3) OUR GOAL is to determine so called "ox tox trigger zones" where seizures originate during exposure to HBO and then determine the effects of mitochondria-targeted antioxidant therapy on this neural activity using MitoQ. Two to 4 intracerebral recording microelectrodes (tungsten; FHC, Inc.) are implanted using the stereotaxic frame/manipulator into motor cortex and deep subcortical nuclei that are implicated in seizure genesis (e.g., brain stem cardiorespiratory nuclei, cranial nerve nuclei, or medullary reticular formation) and seizure amplification/propagation nuclei (e.g., CA1 hippocampus, thalamus, or hypothalamus). Up to four recording electrodes can be implanted in the brain in the rat during sterile recovery surgery. Recording and reference electrodes are secured to the skull using dental adhesive. The telemetry module (4ET, Data Sciences International, DSI Inc.) is implanted in the abdomen and the four pairs of biopotential leads are tunneled under the skin, exposed at skull, and attached to the recording microelectrodes and epidural reference electrodes (Weiergräber et al. 2005 Electrocorticographic and deep intracerebral EEG recording in mice using a telemetry system. Brain Res 14(3):154-64; and Papazoglou et al. 2016 Non-restraining EEG Radiotelemetry: Epidural and Deep Intracerebral Stereotaxic EEG Electrode Placement. J Vis Exp. (112): 54216, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4993274/bin/jove-112-54216.mp4).

After recovery from surgery (at least 1 wk), each animal is dosed with either MitoQ, TPMP, or vehicle and dived to 5 ATA O2 (HBO) until onset of 1st and 2nd bouts of seizures as in EXPERIMENT 2. After the first dive and bout of seizures, at least one week later, the animal is treated with one of the other two substances and dived again until onset of 1st & 2nd bouts of seizures. The order of treatments is randomized across two dives in animals; however, each rat is treated with only two substances and only dived twice, thus there are three test groups: MitoQ & TPMP, MitoQ & Vehicle, and TPMP & Vehicle. Each electrode records field potentials; i.e., neural activity from many neurons and synapses concurrently as their summed electrical activities. Recordings made in the brain stem (medulla oblongata, pons or midbrain) will be called an "electrobulbogram (EBG)"; in the thalamus or basal ganglia (caudate, putamen, globus pallidus & substantia nigra) will be called a deep "electroencephalogram (dEEG)"; and in the motor cortices it will be called an "electrocorticogram (ECoG)". All experiments are done in unrestrained, freely behaving animals. The length of time spent in

the animal chamber inside the hyperbaric chamber is no longer than 2h, 45min. Each animal has access to 5-6 pieces of food kibble lying on the bottom of the chamber and hydrogel. A PILOT STUDY will be done initially to develop the deep intracerebral microelectrode recording capability, which we estimate will require up to 10 animals. The actual study will use 10 rats/group: [(10 rats/group) x (3 groups: MitoQ/TPMP, MitoQ/vehicle, TPMP/vehicle) x 1.15 c.f.] + (10 rats for pilot study) = 45 rats.

END OF WHOLE ANIMAL RAT EXPERIMENTS

BEGIN RAT BRAIN SLICE EXPERIMENTS

EXPERIMENT 4) Determine the effects of MitoQ during exposure to hyperoxia on the rate of production of reactive oxygen and nitrogen species and mitochondrial membrane potential in brain stem neurons in tissue slices. Brain slices are harvested from rats ranging in age from postnatal day 1 through 21 (P1-P21), weanlings, and young juveniles to young adults. For brain slice experiments to measure reactive species and mitochondrial membrane potential we estimate the following numbers of rats: Assumptions and power analyses used to determine the number of cells needed and animals needed for fluorescence imaging are explained below in Section 9. To measure reactive species: [(40 cells/treatment) x (6 rats/40 cells) x (2 dyes) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 40 rats; and to measure mitochondrial membrane potential: [(40 cells/treatment) x (6 rats/40 cells) x (1 dye) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 20 rats

EXPERIMENT 5) Determine the effects of MitoQ on cellular O2-sensitivity using electrophysiology in rat brain slices. Brain slices are harvested from rats as in experiment 4. Estimated numbers of animals required are calculated as follows: [(10 slices/treatment) x (1 rat/cell) x (2 treatments: hyperoxia, MitoQ) x (1.4 c.f. x 2.2 c.f.) x (1 CNS nucleus)] = 62 rats

In Section 9, we explain allowances for rat pups born but not used in experiments (13 rats, neonates & weaned rats born but not used in this research (too old for certain experiments)) and the damns required for pups (12 rats, timed-pregnant or nursing dams). In all, 135 rat pups need to be born to yield 40 + 20 + 62 = 122 rat pups for experiments.

END OF RAT BRAIN SLICE EXPERIMENTS

BEGIN AQUAPORIN-4 (AQP4) MOUSE EXPERIMENTS (no MitoQ or TPMP will be used in these experiments, only HBO)

AQP4-KO and WT control mice will be shipped to USF Tampa from Case Western Reserve University, Cleveland, Ohio, Dept. of Physiology and Biophysics. Mice will initially be held in quarantine for 28 days in the COM vivarium before they are used in any experiments. CWRU will have a subcontract with USF on the USF Grant from ONR. At present, no MOU or MTA has been established.

EXPERIMENT 6) Effects of HBO on the risk for developing HBO-induced seizures in AQP4-KO vs. WT mice. After quarantine and acclimation to their new holding room, and being housed inside the animal & dive chambers, members of the Dean Lab will subject individual WT vs. AQP4-KO mice to HBO as in Experiment 2, above (rat chow & hydrogel are provided as in Expt. 2). Likewise, as in Experiment 2, no radio-telemetry is used. Visible seizures are scored using the behavior criteria. Each mouse will be dived four times to the point of 1st and 2nd bouts of visible seizures, one dive/week, over a 21-day period;

e.g., 1st Monday (day 1), 2nd Monday (day 7), 3rd Monday (day 14), 4th Monday (day 21). Mice are returned to the COM vivarium between dives. THE RATIONALE FOR MULTIPLE DIVES AND SEIZURES is to induce kindling in mice and determine the effect of AQP4 on kindling. The number of mice required for this experiments is: (10 mice/group) x (2 groups: AQP4-KO & WT) = 20 mice total (10 AQP4-KO and 10 WT)

EXPERIMENT 7) Effects of HBO -/+ CO_2 retention on the risk for developing HBO-induced seizures in AQP4-KO vs. WT mice. As in Experiment 6 only now each mouse is dived twice, at least 1 wk apart, to the point of 1st and 2nd bouts of seizures (rat chow & hydrogel are provided as in Expt. 2). The order of HBO without CO_2 and HBO with CO_2 will be randomized between mice. Mice are returned to the COM vivarium between dives. The number of mice required for this experiments is: (10 mice/group) x (2 groups: AQP4-KO & WT) = 20 mice total (10 AQP4-KO and 10 WT)

EXPERIMENT 8) Effects of hyperbaric oxidative preconditioning on AQP4 expression and the risk for developing HBO-induced seizures in WT mice. Prior to being dived to 5 ATA HBO, each mouse is pretreated (preconditioned) with a sublethal level of HBO (non-seizure promoting: 2.5 ATA HBO x 90-min) four times, interspersed by a 4.5 h room air breathing break, over a 24h period. Two mice are preconditioned at a time with access to food and hydrogel. All animal exposures are done in a Sechrist Animal Research Chamber (Model 1300B, MWP = 35 psig). The pressure chamber is ventilated continuously with air supplied from a compressed cylinder during the 4.5 h interval between 90-min exposures to 2.5 ATA HBO.

THE RATIONALE FOR HBO PRECONDITIONING DURING 24 HOUR PRIOR TO DIVING IS TO UPREGULATE AQP4 EXPRESSION IN THE BRAIN. After the 4th 90-min exposure to 2.5 ATA HBO, the mouse is kept on 1 ATA air for 4.5 h inside the preconditioning chamber. After that, the mouse is transferred to dive chamber (in the same room) and held on 1 ATA air (15min); Hydrogel and food pellets are placed on the floor of the animal chamber. Next, the ventilating gas is changed to 1 ATA O_2 (15min) at which time the mouse is dived to 5 ATA HBO until the onset of 1st and 2nd bouts of seizures. After the 2nd bout of seizures, the mouse is decompressed to room pressure on air and anesthetized using isoflurane. The mouse remains on HBO up to 2 h or until onset of 2nd bout of seizures, whichever comes first. The total time spent inside the dive chamber is 2.5 h. A cardiac puncture is taken to measure the redox status of the animal using the redoxSYS analyzer (40uL blood/sample) and the perfused transcardially with buffer followed by paraformaldehyde fixative for immunohistochemical analyses of AQP4 expression in the brain. Three groups of WT mice will be studied; 1) mice preconditioned using 2.5 ATA HBO; control WT mice preconditioned with normoxic hyperbaric pressure (14% O_2 + balance Helium = 0.21 ATA O_2 + 0.79 ATA He), and another group of control WT mice maintained in the preconditioning chamber for 24h with ventilation using 1 ATA air. For example, the experimental protocol would be to initiate preconditioning at 1) 8am-9:30am, 2) 2pm-3:30pm, 3) 8pm-9:30pm, 4) 2am-3:30am, and then dive in HBO at 8am on next day. A member of the lab team will remain in the lab with the animal during the entire 24 h preconditioning period. The number of mice needed for this experiment is: (3 groups of preconditioned WT mice: HBO, normoxic hyperbaric pressure, and 1 ATA air) x (10 mice/group) = 30 WT mice

BUMED Animal Use Appendix for Research Involving Animals

ABBREVIATED VERSION

*****NOTE: BUMED will ONLY review protocols that have been approved by your IACUC.*****
****Animal work MAY NOT be initiated until the awardee receives BUMED approval.****
Animal work initiated without BUMED approval is noncompliant and may not be funded.

Institutions using DoD funds to support the use of animals in research, product development, testing and education projects must provide electronic copies of the following documents to the Department of the Navy (DON) Bureau of Medicine and Surgery (BUMED) Veterinary Affairs Office for review and approval prior to initiation:

a. A copy of their **IACUC-approved institutional protocol(s)** (BUMED will ONLY review **approved** protocols) and documentation of IACUC approval

b. A copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by BUMED **PRIOR** to implementation)

c. A completed Appendix for each IACUC-approved protocol.

This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs.

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DoD regulations. The DoD policies and requirements for the use of animals in DoD-supported research, development, testing and evaluation are described in DoD Instruction 3216.01, dated September 13, 2010 and SECNAVINST 3900.38C, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to BUMED Veterinary Affairs:

Phone: [redacted]
Email: DON – VRPP, [redacted]
Mail: Department of the Navy Bureau of Medicine and Surgery
ATTN: Director for Veterinary Affairs
Bldg 1564 Room 122
1564 Freedman Drive
Fort Detrick, MD 21702

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Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text. Submit electronic copies of the appendix only; please do not submit printed copies to BUMED. Any section of the Appendix that is not applicable to your proposal, e.g., no surgery or no prolonged restraint, should be marked "**No**" or "**N/A**". There are no space limitations for the responses.

It is essential that only animal studies or procedures documented in an IACUC–approved protocol or amendment be performed at your facility. BUMED will collect animal usage information for end-of-year DoD animal use reports separate from the grant annual progress reporting requirement throughout the life of your award. For this reason, Principal Investigators or other delegated research personnel should keep accurate records and be able to provide an audit trail of all animal use that correlates to their approved protocol.
~~~~~~

[X] Yes [ ] No All animal studies described in the attached IACUC-approved protocol are funded by this DOD award/contract. If no, STOP ! You must use the **full version** of the Animal Use Appendix.

1. Administrative Data: (Provide Attending Vet, IACUC, and Research Office info for the work site.)

DoD/Contract PI Name: Stephen R. Thom, MD, PhD

Grant/Contract PI Email: (b) (6) [redacted] **Phone:** (b) (6) [redacted]

Protocol PI Name: Stephen R. Thom, MD, PhD

Protocol PI Email: (b) (6) [redacted] **Phone:** (b) (6) [redacted]

Animal Research Site (RS): University of Maryland School of Medicine

RS Attending Veterinarian: (b) (6) [redacted]

Attending Vet Email: (b) (6) [redacted] **Phone:** (b) (6) [redacted]

RS IACUC Point of Contact (POC): (b) (6) [redacted]

IACUC POC Email: (b) (6) [redacted] **Phone:** (b) (6) [redacted]

RS Grants Office Point of Contact (POC): (b) (6) [redacted]

Grants Office POC Email: (b) (6) [redacted] **Phone:** (b) (6) [redacted]

Animal Protocol Title: Neuroinflammation and glymphatic dysfunction in decompression sickness

2. Total Number of Animals Used (by Species) and USDA Pain/Distress Category:

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Mouse (Mus Musculus)	B	36
Mouse (Mus Musculus)	C	616
Mouse (Mus Musculus)	D	1528

USDA Pain/Distress Category Definitions:

- ***Column B:*** *Animals being bred, and animals being held for use in research, testing, teaching, experiments or surgery but not yet used for those purposes.*
- ***Column C:*** *List the number of animals that will experience no more than slight or momentary pain or distress as a result of experimental manipulations or procedures on this protocol.*
- ***Column D:*** *List the number of animals that will potentially experience more than momentary or slight pain or distress that **WILL** be alleviated through the use of anesthetics and/or analgesics.*

- ***Column E:*** *List the number of animals that will experience more than momentary or slight pain or distress that* ***WILL NOT*** *be alleviated or relieved with anesthetics or analgesics.* **If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:**. .

3. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals. Note the DoD-specific database requirements in subparagraph a.

a. Literature Source(s) Searched:
http://www.ntis.gov/products/fedrip.aspxhttp://projectreporter.nih.gov/reporter.cfm

[X] Federal Research in Progress (FEDRIP) http://www.ntis.gov/products/fedrip.aspx

or

[X] Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER)
http://projectreporter.nih.gov/reporter.cfm

b. Date of Search: February 19,2022

c. Years Covered By Search: 1985 - 2022

d. Key Words and/or Search Strategy: Decompression sickness (DCS) and animal model. Decompression and bubbles, Decompression and microparticles (MPs), Decompression and glymphatics, Glymphatics and pressure, Glymphatics and microparticles

e. Results of Search: To our knowledge, there are no other groups performing similar research as we are planning. Our database search resulted in 103 hits; 89 were not duplicative as they were not pertinent to inflammatory changes or microparticle processes and none were investigating glymphatic function. Among 14 hits pertaining to glymphatic function none related to high pressure or decompression stresses.

****Questions 4-10 refer to the Research Site and Protocol Principal Investigator.****

Information and/or documents required in questions 4-8 should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to BUMED by the Protocol Principal Investigator (PI) or institution staffs. If the Protocol PI prefers that BUMED staff contact the institution to obtain this information and/or documents, the PI must specifically request this action in writing. The Protocol PI should contact BUMED (see contact information on cover page) to arrange for submission of this written request.

4. Institutional Animal Care and Use Committee(s) (IACUC) Approval(s):

Institutional Animal Use Protocol Number:	0222008		
IACUC (initial) approval date:	March 11, 2022	Protocol expiration (rewrite) date:	February 18, 2025

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

Animals are housed and veterinary care provided at the University vivarium. Mice are kept in small cages, typically 4 -8 per cage depending on size/age in temperature- and light/dark-cycle

controlled rooms. Technicians provide daily monitoring (including weekends and holidays) for food and water *ad libitum* with full-time veterinarians on staff who are present during normal working hours and on-call for weekends and holidays to provide any emergency care.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

[X] Yes [ ] No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

[ ] Yes [X] No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

[ ]	Yes	[X]	No	Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Stephen R. Thom (b) (6) February 19, 2022

(Protocol Principal Investigator Printed Name) (Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

New Animal Use Protocol (AUP) Submission Checklist

This checklist has been prepared to assist PIs with a new AUP submission. Please check off each relevant item applicable to this protocol. The protocol will not be accepted without this form. Additionally, incomplete protocols will be returned.

☒ Animal Use Protocol (AUP) Form (*Version Date: 02/2021*)

☒ All Species (x NHP) Enrichment / Socialization Form (*Version Date: 02/2021*)

☐ Non-Human Primate (NHP) Enrichment / Socialization Addendum (*Version Date: 02/2021*)

☒ Electronic copy of funded grant(s) supporting this work (*Emailed to IACUC on:* Click here to enter a date.)

☒ Rodent Breeding Addendum (*Version Date: 10/2021)*

☐ Hazardous Agent Addendums – Pathogenic Organisms, Radioactive Materials & Other (*Version Date: 09/2020*)

☐ Hazardous Agent Addendums – Chemical Level 1 or Chemical Level 2 (*Version Date: 09/2020*)

☐ PI-Managed Satellite Housing Facility Addendum (*Version Date: 02/2021*)

☐ IBC Approval Letter or IBC application status: _____

☐ RSC Approval Letter or RSC application status: _____

☐ ESCRO Approval Letter or ESCRO application status: _____

☐ UMMC Administration Approval Letter or UMMC review status: _____

☒ Survival Surgery Consult **ALL SPECIES**
Date of Consult (*within last 3 months*): Feb 2, 2022
Veterinarian Consulted: (b) (6) [redacted]

☐ Non-survival Surgery Consult **USDA regulated species only**
Date of Consult (*within last 3 months*): _____
Veterinarian Consulted: _____

☐ BVAMC Attending Veterinarian Consult
Date of Consult (*within last 3 months*): _____
Veterinarian Consulted: _____

☐ BVAMC SRS Approval or RPSS application status: _____
****NOTE: A new RPSS must be submitted with each new IACUC protocol submission.****

I certify that all required components of a new AUP submission (*as applicable*) are attached and complete.

SRT
PI or PI Designee Name (printed)

OAWA Internal Use:

____ All Relevant Items Attached
____ Current versions utilized
____ Adherence to AUP format
AUP Date Rec'd: _____

IACUC # _____

OAWA Staff Initials: _____
Date: _____

UNIVERSITY OF MARYLAND BALTIMORE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
-- ANIMAL USE PROTOCOL --

Principal Investigator: Stephen R. Thom Degree: MD, PhD Employee ID#: _____
Campus Mail Address: (b) (6) Dept: (b) (6)
E-mail: (b) (6) Office Phone: (b) (6) Emergency Contact # *(24 hr access)*: (b) (6)
Secondary Lab Contact: (b) (6) Email: (b) (6) Phone: (b) (6)

AUP Title: Neuroinflammation and glymphatic dysfunction in decompression sickness

I. RESEARCH CATEGORIES
Major Categories of Research: Please check all that apply.

☐ Antibody Production
☒ Behavioral Studies
☒ Breeding *(*attach breeding protocol as an addendum to the experimental protocol. Breeding template on OAWA Website)*
☐ Chronic or Prolonged Restraint
☒ Exogenous Substances *(e.g., any substance administered to an animal except anesthetics or analgesics)*
☒ Euthanize and Harvest Tissue
☐ Food / Fluid Regulation
☐ Hazardous Agents
☒ Imaging
☐ Investigational Device
☐ Irradiation
☒ Non-Survival Surgery
☐ Oncology / Tumor Production
☐ Physiologic Measurements *(e.g., blood pressure, telemetry, electrophysiology, etc.)*
☐ Sampling (*e.g., blood, urine, bone marrow, biopsies, etc. – collected antemortem*)
☐ Satellite Housing *[e.g. animals maintained outside animal facility >12 hours (USDA) or >24 hours (rodents)]*
☐ Specialized Diet
☐ Specialized Husbandry (*e.g., Singly housed animals, BSL3 housing, metabolic cages, reverse light cycle, etc.*)
☒ Survival Surgery
  ☐ Multiple Survival Surgery (*e.g., major + minor surgeries or multiple minor surgeries in one animal*)
  ☐ Multiple *Major* Survival Surgery (*e.g., opening body cavity more than once in one animal)*
☐ Teaching / Training
☐ Use of Hospital Equipment [(*e.g., imaging or radiation producing equipment) *refer to Use of Hospital Equipment Guidelines on OAWA Website*]

Will the animal work proposed in this protocol be carried out under Good Laboratory Practices (GLP) in support of, or intended to support, applications for research or marketing permits for products regulated by the Food and Drug Administration (FDA) or the Environmental Protection Agency (EPA), e.g., human and animal drugs, medical devices for human use, etc.? ☐ YES or ☒ NO
If YES, please contact the SOM GLP Office (b)(3)[Title 10 USC 130b], (b) (6) *for more information.*

II. CATEGORY OF PAIN OR DISTRESS
Only the highest category of pain or distress should be indicated.

☐ CATEGORY B – Animals bred, conditioned or held for use in teaching, testing, research, but not yet used for such purposes. (*NOTE: If tail snips are necessary for genotyping, this category is not appropriate.*)

☐ CATEGORY C – Procedures that involve no or only very brief pain or distress, with no need for or use of pain relieving drugs.

☒ CATEGORY D – Procedures involving potential pain or distress for which appropriate anesthetics, analgesics, or tranquilizers are given. *(NOTE: Contact a Veterinary Resources (VR) veterinarian for "appropriate" agents as needed)*

☐ CATEGORY E – Painful or distressful procedures for which drugs to relieve the pain or distress would adversely affect the research study. *Under 'Detailed Description of Animal Procedures' in the written protocol, please include subheading Category E Justification and provide strong scientific justification for conducting procedures that involve unrelieved pain or distress. Refer to the last page for guidance.*

III. FUNDING SOURCE (*CURRENT*)
Provide one copy of each funded grant for IACUC file. At a minimum, the Research Methods and Vertebrate Animal Section should be included with the submission.

- ☐ US Public Health Service (e.g., NIH)
- ☐ Department of Veterans Affairs
  - ☐ This research is funded by the Department of Veterans Affairs, or
  - ☐ This research is administered through the Baltimore BREF
- ☐ Private or Charitable Foundation
- ☐ Private Company
- ☐ Departmental Funds
- ☐ Other

Sponsor / Agency	Grant Identification Number*	Grant Title
Office of Naval Research	**N00014-21-S-B001**	Neuroinflammation and glymphatic dysfunction in decompression sickness

Please add rows to these tables by placing cursor at end of last row and clicking return.

Does the PI or any of the research staff have a financial interest related to the proposed research or the sponsor that must be disclosed accordingly to UMB's Conflict of Interest in Research and Development and UMB's Policy and Procedures on Financial Conflict of Interest to Promote Objectivity in Public Health Service-Funded Research?

☐ YES or ☒ NO

If YES, please contact the Research Integrity Office and speak with (b) (6) [redacted].

IV. EXPERIMENTAL ANIMAL USAGE
Each species (*by age*) should be listed separately. This table is for adult and/or juvenile (*post-weaning*) experimental animals only. ***Strain designations and the number of breeders are no longer required in this table. # of breeders should be provided in the Breeding Addendum only.*

Species	Age	Weight range *(USDA Covered Species Only)*	Sex	Total # *(for 3 yrs)*	Max. Daily Census*
Mouse	☒ Juvenile (*weaned*) or Adult (n=2144) ☐ Timed pregnant ☐ Dams w/ pups ☒ Other - *Specify*: 36 breeders		☐ Male ☐ Female ☒ Both	**2180**	6
	☐ Juvenile (*weaned*) or Adult		☐ Male		

	☐ Timed pregnant ☐ Dams w/ pups ☐ Other - *Specify*:		☐ Female ☐ Both		
	☐ Juvenile (*weaned*) or Adult ☐ Timed pregnant ☐ Dams w/ pups ☐ Other - *Specify*:		☐ Male ☐ Female ☐ Both		

*Max. Daily Census = approximate number of animals to be housed in the facility on any given day. Please note that this information is necessary to plan for housing space and should be expressed in whole numbers.
***Please add rows to these tables by placing cursor at end of last row and clicking return.*

Will embryonic and/or pre-weaning animals be used at any time during this protocol? ☐ YES ☒ NO
If yes, state species, age and number required for 3-year protocol (estimations based upon species characteristics or lab experience are acceptable).

Species	Age *(days)*	Total # *(for 3 yrs)*

Will additional animals be required for training current or new staff on proposed animal procedures? ☐ YES ☒ NO
If yes, state species, age and number required for 3-year protocol. Under 'Detailed Description of Animal Procedures' in the written protocol, please discuss training procedures.

Species	Age *(days)*	Total # *(for 3 yrs)*

V. HAZARDOUS AGENT(S) IDENTIFICATION

Please contact EHS for assistance as needed. *Link to Hazardous Agent Addendums.*

Will pathogenic organisms be utilized in live animals in this protocol? ☐ YES ☒ NO
If yes, complete a hazardous agent addendum for each pathogenic organism.

Will recombinant DNA be utilized in live animals in this protocol? ☐ YES ☒ NO
If yes, complete a hazardous agent addendum for each category, e.g., rDNA, plasmids, siRNA, viral vectors, recombinant cells, etc.

Will radioactive materials be utilized in live animals in this protocol? ☐ YES ☒ NO
If yes, complete a hazardous agent addendum for each radioactive material.

Will hazardous chemicals be utilized in live animals in this protocol? ☐ YES ☒ NO
If yes, complete a hazardous agent addendum for each chemical level, e.g., CL1 or CL2.
Please refer to EHS Hazardous Chemical Use in Animal Research website for guidance.

VI. METHOD OF EUTHANASIA

Please list each method of euthanasia to be used in this protocol. Please refer to the Recommended Methods of Euthanasia located on the OAWA Website under Guidelines. *Method and description can be cut and pasted into the below table.*

SPECIES	METHOD	DESCRIPTION
Mouse	Inhalant anesthetic overdose followed by exsanguination, perfusion with saline then paraformaldehyde and major organ harvest	Using a precision vaporizer with induction chamber and waste gas scavenger, Isoflurane, in oxygen, will be administered slowly at >4.5 % and continued until respiratory arrest occurs for > 60 seconds. Isoflurane is stopped, the chamber is flushed with oxygen only, the animal is removed from chamber, rapidly exsanguinated by cardiac puncture or thoracotomy and using a chemical fume hood, then perfused with saline followed by

		paraformaldehyde for brain harvest and immunohistochemical analysis. Excess perfusate will be collected and EHS contacted for disposal.
Mouse	Post-Ketamine/xylazine anesthesia followed by exsanguination	IP Ketamine (80-150 mg/kg), xylazine (8-15 mg/kg). Injectable anesthetic administered IP using a 25 gauge needle to a level of surgical anesthesia followed by exsanguination and organ harvest.

***Please add rows to this table by placing cursor at end of last row and clicking return.*

VII. LOCATION OF ANIMAL USAGE

BUILDING / RM # *List each location only once.*	LAB	ANIMAL FACILITY	LIST ALL PROCEDURES TO BE PERFORMED IN LIVE ANIMALS IN EACH LOCATION *(e.g., surgery, injections, behavior, imaging, euthanasia, etc.)*
BRB 4-015	☒	☐	Surgery, injections, decompression stress, behavior, euthanasia, perfusion
HH 685	☐	☒	Animal husbandry
HH645	☒	☐	MRI imaging studies
BRB 4-4039A	☒	☐	Behavioral studies
	☐	☐	

***Please add rows to this table by placing cursor at end of last row and clicking return.*

VIII. CONSIDERATION OF ALTERNATIVES TO POTENTIALLY PAINFUL / DISTRESSFUL PROCEDURES
Keep copies of computer database search results in your protocol file for a minimum of three years after the protocol has expired to demonstrate your compliance with the law if regulatory authorities or the IACUC should choose to audit your project. *Refer to instructions for further information regarding the completion of this section.*

1. Do any of the following criteria apply to this animal use protocol? ☒ YES ☒ NO
   If yes, check all that apply and complete question 2. If no, STOP questions 2-6 are not required!

   ☐ USDA Covered Species (*any species other than mice, rats, birds, fish, amphibians or reptiles*)
   ☐ Pain / Distress Category E
   ☐ Funded by the VA
   ☒ Funded by the DoD

2. Does this protocol include procedures that have the potential for producing more than momentary or slight pain or distress? ☒ YES ☐ NO *If yes, complete questions 3-6. If no, STOP questions 3-6 are not required!*

3. What are the potentially painful procedures or distressful conditions proposed in this animal use protocol?

   Survival surgery (ligation od lymphatic channels), decompression sickness

4. A literature search for alternatives to the potentially painful or distressful procedures (*listed above*) was performed. Search methods and sources are documented in table below:

Name of Database Searched: (*Minimum of 2*)	**Date search was performed:** (*mm/dd/yy*)	**Years covered by the search:** (*from yyyy to yyyy*)	**Search Strategies used:** (*including scientifically relevant terminology and Boolean operators*)	# of hits:	# of relevant hits:
FEDRIP	2/19/22	1085-2022	Decompression sickness and animal model	89	9

			Decompression sickness and alternative	0	
			Decompression sickness and glymphatics	0	
			Lymphatic ligation and glymphatics	14	6
			Lymphatic ligation and alternatives	0	
RePORTER	02/19/22	1985-2022	Decompression sickness and animal model	43	6
			Decompression sickness and alternative	0	
			Decompression sickness and glymphatics	0	
			Lymphatic ligation and glymphatics	12	4
			Lymphatic ligation and alternatives	0	

Database Resources: UMB HS-HSL Databases and AWIC

5. Did the literature search reveal less painful or distressful alternatives to the potentially painful or distressful procedures that are proposed?

   ☒ No alternatives, refinements, replacements or reduction methods were found.

   ☐Yes, but they cannot replace the procedures that are proposed. *See below explanation*.

   a. If yes, identify the painful or distressful procedures(s) and provide a BRIEF explanation why the alternatives found to this potentially painful or distressful procedure(s) were not acceptable alternatives.

      Click here to enter text.

6. List other sources of information used to refine this protocol, as applicable.

   Click here to enter text.

PRINCIPAL INVESTIGATOR'S ACKNOWLEDGEMENT OF RESPONSBILITY

I acknowledge and accept the responsibilities associated with serving as the principal investigator of an IACUC protocol as delineated in the OAWA Principal Investigator Manual.

I will conduct the project in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), and the *Guide for the Care and Use of Laboratory Animals*.

I authorize individuals listed on this application to conduct procedures involving animals and I accept responsibility for their oversight in the conduct of this proposal.

I confirm that all individuals working with animals have completed the IACUC required training and are participating in an appropriate Occupational Health & Safety program. Further, I certify that those individuals are properly trained, or will receive such training prior to working with animals, in all areas relevant to their assigned work with animals (e.g., biology, handling, and care of the species used; aseptic surgical methods and techniques; the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics & analgesics; and procedures for reporting animal welfare concerns).

For animals under this proposal, I understand that in cases of necessary medical treatment, Vet Resources veterinarians are authorized to provide any treatment required to sustain life; or if necessary, provide humane euthanasia to prevent unapproved distress and/or pain. I recognize that the veterinary staff will contact me as soon as possible using the emergency contact information that I provide in this application, but I understand that such contact may not always be possible prior to providing treatment or performing euthanasia.

I will notify the IACUC of unanticipated outcomes of animals use, including protocol or non-protocol disease or injury. Unanticipated outcomes are generally defined as negative impacts to animal welfare or well-being.

I recognize that veterinary consultation must occur when pain or distress is beyond the level IACUC approved in this protocol, or when my staff are unable to provide interventional control (i.e., euthanasia, immediate removal from the

study). I will notify VR veterinary staff when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs.

I will obtain approval from the IACUC before initiating any change in the study design or procedures by submitting an amendment request. I understand that work performed without IACUC approval cannot be published with certification of IACUC approval and may result in federally required reporting of non-compliance.

I am familiar with the University of Maryland Baltimore Environmental Health and Safety (EHS) policies and procedures relative to laboratory safety, biohazards, radiation safety, occupational health and safety, laser safety, chemical and biological waste management practices, select agents, and shipping of infectious materials and clinical specimens. I will abide by the regulations, policies and procedures that relate to research, testing or training at UM. I will ensure that all laboratory personnel engaged in this project will be informed of potential hazards and adequately trained in procedures of animal experimentation involving hazardous agents.

___via email _____ _3/8/2022_
Signature Date

__Stephen R Thom_____
Printed Name

_____
Signature – VA Attending Veterinarian *(if animal work is funded by the VAMC or conducted in the VAMC)*

WRITTEN PROTOCOL

Lay Summary *(limit response to ¼ - ½ page)*:

1. Using non-technical (lay) language, please describe the purpose of the proposed study.

Decompression sickness (DCS) presents a health risk for compressed air divers and caisson/tunnel workers, and it poses a constraint to provocative diving and submarine escape. Bubbles play a role in pathophysiological responses mediating DCS, however, they are often produced without symptoms. Therefore, additional etiological factors have been sought to explain DCS development. Microparticles (MPs, small vesicles present in the circulation) are elevated in association with simulated as well as *bona fide* underwater diving. These MPs carry inflammatory proteins that damage blood vessels throughout the body, including those in the brain. Maneuvers which decrease the incidence of DCS in the animal model also diminish MPs production in human divers. As MPs cause vascular damage and may also participate in gas bubble nucleation (a required stimulus to form bubbles in the body), their formation presents a feasible explanation for most aspects of DCS pathophysiology. Hence, further research focused on MPs has direct relevance to U.S. Navy operational diving, commercial, scientific and sport divers whose activities are constrained by DCS risk. The only meaningful intervention that has come from decades of traditional DCS research is staged decompression tables to limit symptom development. This project will take a novel approach to identify the role of neuroinflammation and MPs in DCS and further explore the potential use of a pharmaceutical agents that we have found to abrogate MPs injuries in other disorders. These might prove to be useful as clinical prophylactic and therapeutic interventions.

2. Describe the potential scientific benefit of the proposed study with respect to human and animal health, the advancement of knowledge, or the good of society.

These studies will improve understanding of decompression sickness and its treatment. While previous studies by us and others suggest that pressure exposure followed by decompression causes an adaptive immunological response, the results do not explain early activation of blood-borne white cells (neutrophils), the predilection of neutrophils for attacking the blood vessel lining of the brain and elsewhere, or why functional deficits are manifested over hours to days after the exposure. Another unexplained observation is the ability to transmit the decompression-induced insult to naïve mice by injections of microparticles isolated from the blood of decompressed mice. These are the specific points that will be addressed in this project.

Justification for the use of animals and species (*limit response to ¼ - ½ page*):

1. Please justify the use of animals in general. Discuss whether other alternatives (*e.g., cell culture, computer modeling/simulation*) to animal usage exist and if so, why they are not feasible, etc.

Currently, no non-animal model of decompression sickness can effectively reproduce the effects of brain injuries seen in animal and human exposures. Mice are well characterized in the laboratory and have been chosen to produce the most widely used animal brain injury models allowing adequate breeding, use of specific knock-out mice, surgical and post-surgical physiologic monitoring. Mice have been widely used in the studies of many brain injury including carbon monoxide, carbon dioxide, hypoxia and ischemia, making comparisons between our results and other studies possible. Our lab has well-characterized rat and mouse decompression sickness models. Per our database searches we found no alternative model using a less sentient animal, computer or cell culture model meeting the scientific aims of this submission.

2. Please document why a particular species was chosen for these studies (*cost is not acceptable as the sole justification*). Additionally, please justify the use of single sex / gender animals, e.g., experiments utilizing all males or all females.

We chose C57BL/6J mice because they are the most extensively used species/strain for studies of many brain injuries, including many of our prior studies. Experiments will be performed using equal numbers of male and female mice.

3. **Strain Identification** *(Experimental & Breeder Animals)*

Strain	Brief description of relevance to the proposed research
C57BL/6J	Mouse strain most often used in mouse decompression, CO, hypoxia, traumatic brain injury and stroke models.
B6.129S1-*Cd36*^{tm1Mfe}/J **[aka CD36 KO]**	CD36 knock-out mice exhibit resistance to some ischemia-reperfusion and hypoxia insults. The mice B6.129S1-***Cd36tm1Mfe***/J lack the CD36 protein, a cell-surface pattern recognition receptor, and have been found resistant to brain ischemia reperfusion. Prior work in our lab has shown these mice do not generate inflammatory microparticles because of the CD36 absence on endothelium, astrocytes and microglia. Therefore, we believe these animals will be of major use in this investigation.

***Please add rows to this table by placing cursor at end of last row and clicking return.*

Do any of the above strains exhibit any known phenotypic variations that could potentially affect animal health, well-being or longevity? ☐ YES* ☒ NO
*If yes, please describe:

Do any of the above strains require phenotype induction, e.g., pharmacologically activated, siRNA, viral induced, etc.? ☐ YES* ☒ NO
*If yes, please include induction methods under *Detailed Description of Animal Procedures*.

Experimental Summary / Animal Usage *(limit response to ¼ - ½ page)*:

1. **What are the objectives or underlying hypothesis of the planned experiments? Provide background information on the science. Information provided will be similar to the background section on a grant application but abbreviated to convey what has already been done in the field at large and why these experiments should be done.**

This research project is to evaluate the neuroinflammatory impact of high pressure and decompression using a murine model. It is specifically focused on neurological injuries that occur in approximately 30% of severe clinical decompression sickness (DCS) cases. Upwards of 60% of these manifest global changes that cannot be explained by other processes, such as formation of gas emboli within the blood vessels. The motivation for the project is to establish the molecular basis for neurological DCS. We have found that inflammatory microparticles (MPs) elevated in humans, and in animal models of DCS trigger dysfunction of the brain glial-based lymphatic (aka glymphatic) system in mice. We have identified a complex pathological interaction between brain-generated MPs and systemic inflammation that may resolve several unexplained clinical observations. We have also identified knock-out mice resistant to DCS pathology and a pharmaceutical in use in humans that protects against CNS injuries. Further, a recent population-based study demonstrated that those sustaining DCS have a 5.7-fold increased risk of developing a sleep disorder as a manifestation of CNS injury, Glymphatic dysfunction has been clearly shown to disturb sleep in multiple animal models.

Pharmaceuticals we will use are agents shown in prior work to eradicate circulating MPs and/or antagonize injuries mediated by the inflammatory cargo they carry. Therefore, studies will be done with an agent introduced into the mouse assess how they alter decompression-induced metabolic and glymphatic changes. Finally, we also plan to use knock-out (KO) mice that do not possess the CD36 protein, as we hypothesize that CD36 is required to allow inflammatory signals to be transmitted from blood to the brain. Others have shown that CD36 KO mice are resistant to brain ischemia-reperfusion damage, presumably because of diminished signaling. Pilot studies have shown CD36KO mice do not generate MPs and are resistant to decompression injuries. We hypothesize these KO mice will be useful to discern the

dynamics of communication between blood-borne MPs and transmission of inflammatory signals across the blood-brain barrier.

2. Does any of the proposed research duplicate previous work? ☐ YES ☒ NO

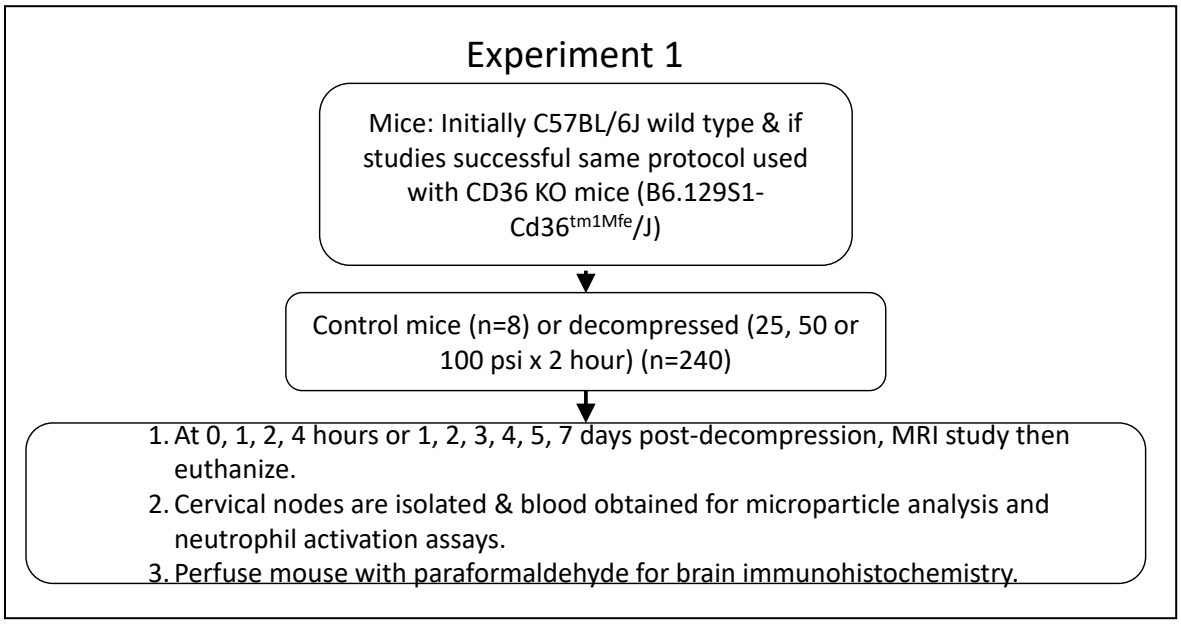
If yes, please discuss the rationale for this duplication.

Click here to enter text.

3. Planned Experiments. *The following information must be provided for each planned experiment.*

Experiment 1 – Brain glymphatic flow assessment by Magnetic Resonance Imaging

Briefly describe the objective of this experiment. Studies will establish the time-course and pattern of glymphatic flow changes after mice are exposed to the standard



decompression stress (100 psi air pressure for 2 hours) as well as graded lower exposures more within established US Navy limits (25 or 50 psi for 2 hours). Note that in rodent studies since 1990 these exposures do not cause animal discomfort. The purpose of these experiments is to assess whether glymphatic dysfunction occurs at lower pressures. Times for studies were chosen based on knowledge of MPs production events. Glymphatic flow in small animal studies is typically assessed using cisterna magna injections of fluorescent tracers. MRI is a potential better method (fewer artifacts) and could be an adjunctive and/or alternative method but needs validation.

Describe what will happen to the animals from start to finish. Mice will be exposed to high pressure for 2 hours in the PI's lab, removed from the pressure chamber and studied at 0, 1, 2, 4 hours, or at 1, 2, 3, 4, 5 or 7 days post-exposure by placing a tail vein catheter for gadodiamide injection. If mice are to be studied more than 4 hours later they will be returned to the vivarium for standard housing until study. At the time the MRI study is to begin, the mouse will have a catheter placed in the tail vein in the PI's lab and then transported for the MRI study to (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6). The animal will be anesthetized in an animal chamber using a gas mixture of O_2 (1 L/min) and isoflurane (>3-4.5 % induction; >1-3% maintenance). The animal will then be placed prone in an animal holder with a nose cone and a bit bar. The surface RF array coil will be positioned over the head of the animal. The animal's eyes will be treated with a petrolatum based ophthalmic lubricant without antibiotics to prevent corneal desiccation. The animal holder will be moved to the center of the magnet and the isoflurane level will be changed to 1.5 to 2 %. The level of isofluorane will be further adjusted based on the respiration rate changes of the animal for the remainder of the experiment. A MR compatible small-animal monitoring and gating system (SA Instruments, Inc., New York, USA) will be used to monitor the animal respiration rate and body temperature. The animal body temperature will be maintained at 35-37°C using a warm water circulation. After moving the mouse on the imaging bed into the magnet, a system calibration will be performed followed by injection of a contrast agent

(gadodiamide) needed for detailed imaging. The IV injection of gadodiamide will be started (0.12 g/kg introduced at 2 µl/minute using an infusion pump) and a series of image acquisitions will followed for the next 2 hours to monitor contrast intake and efflux from the brain. After the study, the animal will be moved out from the magnet and the isoflurane will be stopped. The animal may be returned to the PI lab for euthanasia following the approved protocol. We then obtain brain tissue, blood cells, and cervical lymph nodes for analysis.

What is the longest that any one animal will be involved in an experiment? 1 week

List each experimental and control group, including the group size for each group (n=?). We anticipate that group sizes of 8 mice (4 male, 4 female) will be adequate to overcome variability and allow statistical analysis. There will be 10 experimental groups (0, 1, 2, 4 hours, or at 1, 2, 3, 4, 5 or 7 days post-exposure) and a control group. Therefore, as there are 3 pressure protocols [(3 X 10 groups X 8 mice/grp) + 8 controls] total of 248 wild type mice will be used. We expect that there will be important changes at many of these time points that require mechanistic investigation. Assuming this proves true, the same study pattern will be done repeated with CD36 knock-out mice, as outlined in the Experiment 1 figure.

Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data. Our prior studies looking at MPs responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

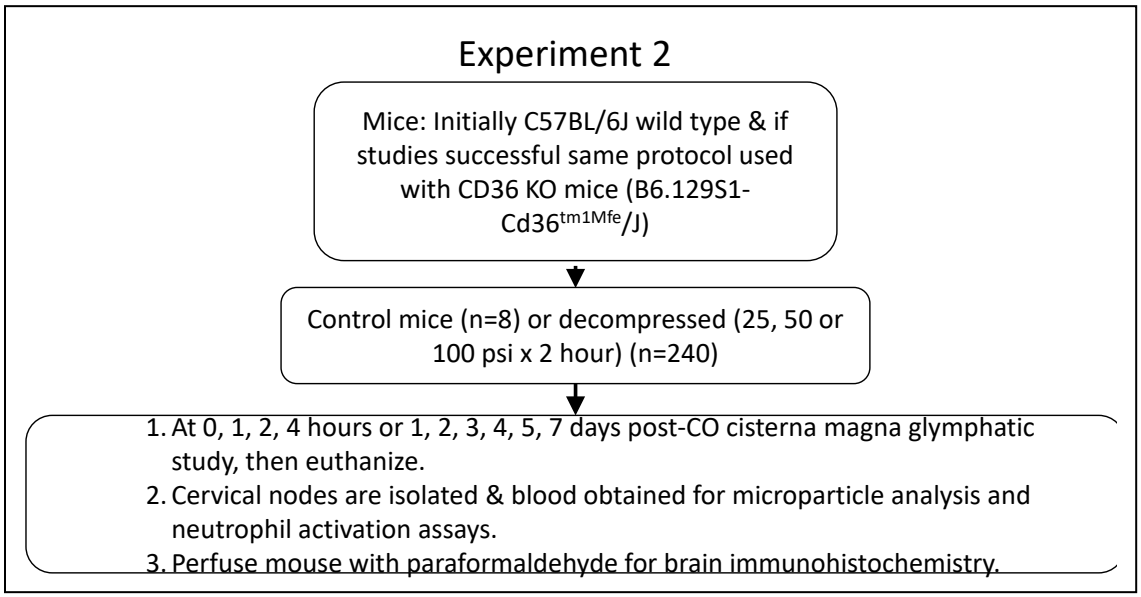
Indicate whether the experiment must be repeated and justify the number of repetitions. No repeats

Indicate the species to be used, and how many will be used *(e.g., X # mice per experiment x X # strains of mice).*
1. Wild type: group sizes of 8 mice (4 male, 4 female), 10 experimental groups and 3 pressure protocols plus a control group. Therefore, a total of 248 wild type mice will be used.

2. CD36 KO: We expect that there will be important changes at many of these time points that require mechanistic investigation. Assuming this proves true, the same study pattern will be done using the CD35 knock-out mice (n=248).

Experiment 2 – Glymphatic flow assessment by cisterna magna fluorescent tracer injections

Briefly describe the objective of this experiment. Glymphatic flow in small animal studies is typically assessed using cisterna magna injections of fluorescent tracers. Additionally this experiment is needed to validate the MRI results. As we need to do histochemical studies, we can couple the histochemical studies (*e.g.* astrocyte and microglial activation, alterations to myelin basic proteins) in the same mice. These studies will be performed entirely in the PI's lab. At the time for study after the pressure/decompression stress, mice will be anesthetized,



and a needle inserted into the cisterna magna. Contrast is injected and then the mouse euthanized at intervals for post-mortem brain analysis.

Describe what will happen to the animals from start to finish. Mice will be exposed to the high pressure/decompression stresses as described in experiment #1 and at time of study, anesthetized with isoflurane and placed in a stereotaxic apparatus that has been cleaned and equipped with a sterile 27guage needle for insertion. After cisterna magna catheter insertion, fluorescent contrast is injected at 2 µl/min for 5 minutes. The mouse will then be monitored continuously while under anesthesia for 15 to 120 minutes before euthanasia by exsanguination so that brain tissue, blood cells, and cervical lymphatics can be studied. The advantage is that using brain sections, fluorescent tracer is quantified and immunohistochemical assessments performed to evaluate perturbation/activation of cells involved with the glymphatic pathway by imaging astrocytes (AQP-4 and GFAP staining), microglia (ED-1 and CD-68 staining), oligodendroglia (native myelin basic protein [MBP] and degraded MBP) and determine their morphological changes as occur with activation and cell injury, and the relationship to cerebral blood vessels (collagen IV and smooth muscle actin staining) following our published methods.

What is the longest that any one animal will be involved in an experiment? 1 week

List each experimental and control group, including the group size for each group (n=?). We anticipate that group sizes of 8 mice (4 male, 4 female) will be adequate to overcome variability and allow statistical analysis. There will be 10 experimental groups (0, 1, 2, 4 hours, or at 1, 2, 3, 4, 5 or 7 days post-exposure) and a control group. Therefore, as there are 3 pressure protocols [(3 X 10 groups X 8 mice/grp) + 8 controls] total of 248 wild type mice will be used. We expect that there will be important changes at many of these time points that require mechanistic investigation. Assuming this proves true, the same study pattern will be done repeated with CD36 knock-out mice.

Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data Our prior studies looking at MPs responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

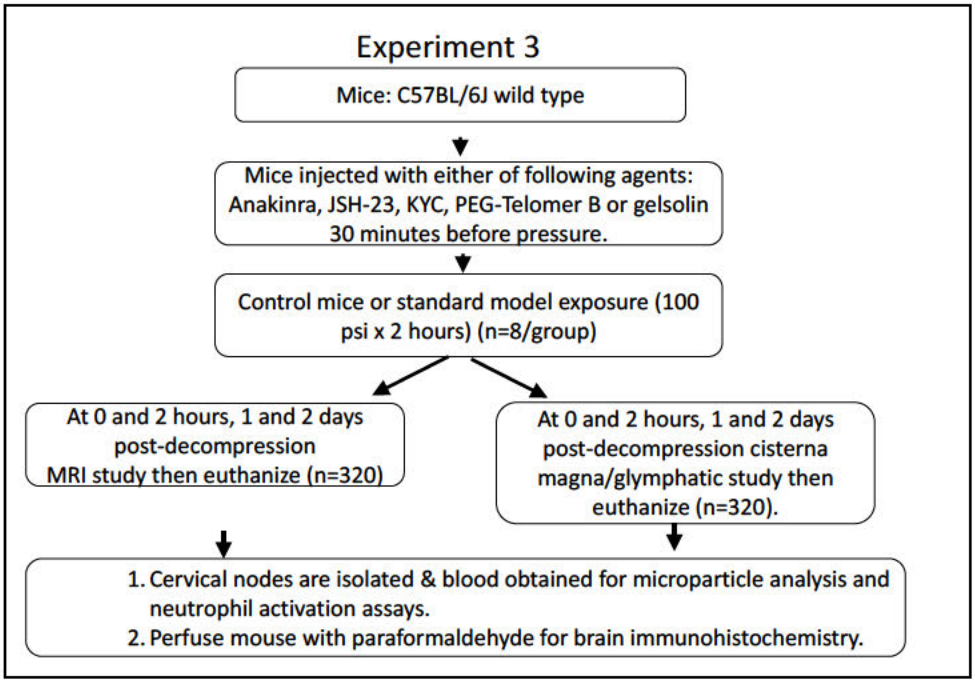
Indicate whether the experiment must be repeated and justify the number of repetitions. None

Indicate the species to be used, and how many will be used *(e.g., X # mice per experiment x X # strains of mice).*
1. Wild type: group sizes of 8 mice (4 male, 4 female), 10 experimental groups and 3 pressure protocols plus a control group. Therefore, a total of 248 wild type mice will be used.

2. CD36 KO: We expect that there will be important changes at many of these time points that require mechanistic investigation. Assuming this proves true, the same study pattern will be done using the CD35 knock-out mice (n=248).

Experiment 3 – Effect of pharmaceuticals on post-decompression glymphatic responses.

Briefly describe the objective of this experiment. The goal of this experiment is to determine whether agents which modify or eradicate MPs in blood will abrogate decompression-induced glymphatic responses. These include PEG-Telomer B, an FDA-approved surfactant that we have shown will lyse blood-borne MPs, human recombinant gelsolin that appears to lyse discretely the inflammatory types of MPs, and also agents that inhibit more classical aspects of inflammatory cascades including JSH-23 (4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine, an inhibitor of the NF-kB pathway), anakinra (an interleukin-1 receptor antagonist), and KYC (acetyl-lysyltyrosylcysteine, an inhibitor of myeloperoxidase). Mice will be injected with agents **30 minutes** ~~either just~~ before ~~or just after~~ exposure to 100 psi air pressure for 2 hours (standard model) to assess effects on inflammatory responses. Mice will be evaluated by MRI and cisterna magna injection studies (described in experiments #1 and #2) at times when neuroinflammatory events (assessed by immunohistochemistry and flow cytometry analysis of cervical node and blood microparticles) are documented. We predict we will need to do studies at times 0-hours, 2-hours post-decompression and days 1 and 2. After euthanasia, we will then obtain brain tissue, cervical lymph nodes and blood for complete analysis. We have based dosages for pharmaceuticals on rodent studies by us and others involved on other forms of neuroinflammation.



Describe what will happen to the animals from start to finish. Mice will be transported to the PI's lab and injected with one of 5 investigational agents. At 30 minutes after injection, they will be exposed to pressure (100 psi X 2 hours) or left as control (n=8). Mice will be studied by MRI or cisterna magna injection glymphatic assessment protocols at four time points: 0, 2 hours, 1 and 2 days post-exposure) and a control group. After the studies mice will be euthanized for brain histochemistry, lymph node and blood analyses. Therefore, a total of 320 mice will be used for each of two study types (MRI or cisterna magna).

What is the longest that any one animal will be involved in an experiment? 2 days

List each experimental and control group, including the group size for each group (n=?).

Control or decompression exposure after injections of one of 5 agents, then study by MRI or cisterna magna injections at 0, 2 hours, 1 day and 2 days post-exposure.

Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data (*e.g., power analysis, reports in the literature, previous experience, etc.*). Our prior studies looking at MPs responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

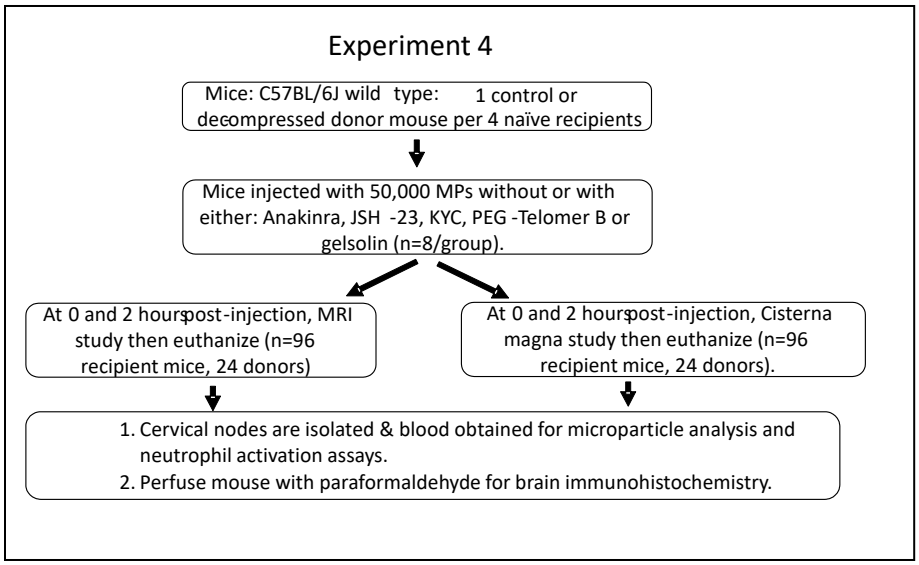
Indicate whether the experiment must be repeated and justify the number of repetitions. none

Indicate the species to be used, and how many will be used Wild type mice: group sizes of 8 mice (4 male, 4 female), control or decompression, study by MRI or cisterna magna injections at 0, 2 hours, 1 and 2 days post-

exposure. Therefore, 8 mice/group X 4 time points x 2 glymphatic analyses X control or decompression X 5 agents = 640 wild type mice will be used.

Experiment 4 – Assess glymphatic flow responses to purified MPs injections.

Briefly describe the objective of this experiment. We want to determine if MPs are the driving force for glymphatic/neurological damage post-decompression. We believe the most definitive approach to achieving this goal is to harvest MPs from blood of decompressed mice and inject them into naïve mice looking to recapitulate tissue injuries. Follow-on studies will then be performed to directly assess the pathological role for inflammatory mediators by co-injecting the MPs along with agents shown effective in experiment 3.



Describe what will happen to the animals from start to finish. Mice will be removed from the vivarium and brought to the lab. Some will be used as donors by exposing them to 100 psi air pressure for 2 hours (or control mice exposed to just air) and euthanizing them at the time of maximum MPs production (we suspect this will be 0 and 2 hours post exposure). MPs will be harvested from cervical lymph nodes, then re-suspended at 50,000 MPs/100 µl sterile PBS. Typically, 1 donor mouse provides sufficient MPs for 4 experimental mice. Recipient mice will be injected via a tail vein with 100 µl solution. Vascular and inflammatory responses to MPs have typically been seen to occur in 1-2 hours in our prior studies, so studies will be done at 0 and 2 hours post-injection (see Figure). These studies will be followed by performing the same experiment but including antagonists shown effective in experiment 3.

What is the longest that any one animal will be involved in an experiment? 1 day

List each experimental and control group, including the group size for each group (n=?). There will be 1 donor mouse for 4 recipient mice. Each time point (0 and 2 hours post-injection) will be done with 8 mice. There will be 6 groups, MPs alone or MPs with one of 5 agents); 8 x 6 = 48 mice x 2 (0 and 2 hours) = 96 recipient mice x study by MRI or cisterna magna injection (2 x 96 = 192 mice). For 192 mice we will need 48 MPs donors (24 control mice, 24 exposed to the 2 hour decompression protocol). Therefore 240 mice in total for experiment 4.

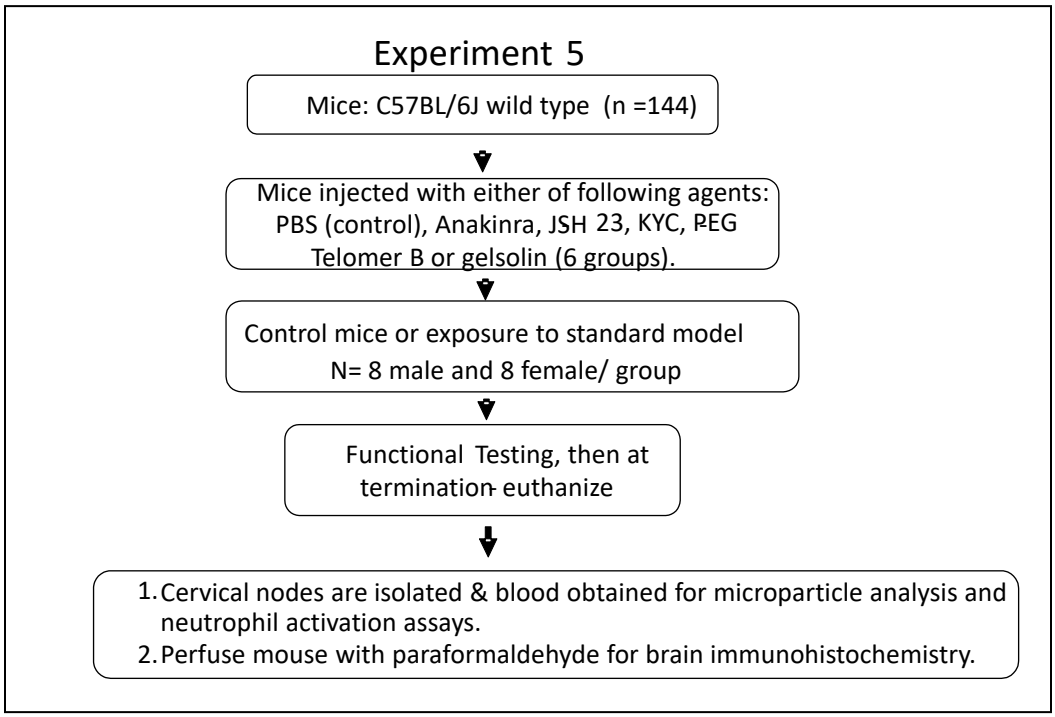
Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data Our prior studies looking at MPs responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

Indicate whether the experiment must be repeated and justify the number of repetitions. No repeats

Indicate the species to be used, and how many will be used. There will be 1 donor mouse for 4 recipient mice. Each time point (0 and 2 hours post-injection) will be done with 8 mice. There will be 6 groups, MPs alone or MPs with one of 5 agents); 8 x 6 = 48 mice x 2 (0 or 2 hours) = 96 recipient mice x study by MRI or cisterna magna injection (2 x 96 = 192 mice). For 192 mice we will need 48 MPs donors (24 control mice, 24 exposed to the pressure protocol). Therefore 240 mice in total for experiment 4.

Experiment 5 – Determine relationship of glymphatic dysfunction with functional deficits.

Briefly describe the objective of this experiment. We will perform functional testing to assess brain-related dysfunction in mice after decompression and determine whether any of the interventions described above that antagonize inflammatory changes mediated by microparticles abrogate the abnormalities. Most tests are simple tasks and we will observe mouse responses. The total time required for the battery of tasks will be **5** days following decompression. The list of tasks are as follows:



1. Novel Objects Recognition Test
2. Beam walking Test
3. Accelerated Roto-rod

Describe what will happen to the animals from start to finish. Each mouse will be utilized in a **5-day** protocol. First, on Monday **mice receive a test agent before pressure exposure (PBS, JSH-23, KYC, PEG Telomer B of gelsolin)** and then exposed to 100 psi for 2 hours. There will also be a control group that will have not received an agent or pressure/decompression. Each testing cycle group will involve 8 mice. Because a number of other studies have shown that sex can have a great impact on learning and behavioral assays, we will be doing studies in a manner to specifically assess responses in each sex. The first sequence will be only male mice: 4 control and 4 pressure-exposed mice. Ensuing cycles will each involve 8 mice, but there may then be all female mice, or comparisons of all pressure-exposed mice but with 4 undergoing one of the interventions (anakinra, JSH-23, KYC, PEG-telomer B, or gelsolin). After these exposures, mice will be taken to our testing room (b) (6) (b) (6) to initiate testing and after each day of testing returned to the vivarium overnight.

These tests are all performed in a quiet room, with indirect lighting inside a Plexiglass arena that will be cleaned with MB10 and manually scrubbed between testing trials. The arena will be given time to dry completely before the next mouse is placed for testing.

Novel Objects Testing will be in the following sequence:
Day 1 (Mon, after the pressure exposure): Acclimation to testing chamber 5 min per mouse
Day 2 (Tues): Acclimation to chamber 5 min per mouse
Day 3 (Wednesday): Novel object recognition test (1 hr retention test) - each mouse is allowed 5 minutes to explore 2 identical objects. They will then be removed to the holding cage for a 1 hour 'retention interval' and then replaced in the testing arena and allowed 5 minutes exploration on 1 old and 1 new object.
Day 4 (Thursday): Novel object recognition test (24 hr retention test).
Day 5 (Friday) each mouse will undergo the Novel object location test.

Beam Walk will be in the following sequence:
Day 1 (Mon, after the pressure exposure): Acclimation to testing room and beam walking apparatus (5 min/mouse).

Day 2 (Tues): Fine motor coordination will be evaluated. A soft padded surface will be placed at the base of the apparatus to cushion any mice that fall. The ability of the mouse to walk across a 1 m beam (3cm, 2cm, 1cm width) will be assessed, measuring distance and time.
Days 3-5: Testing will be repeated daily to evaluate persistence of deficits (recovery has been described in some studies).

Accelerating rotarod will be in the following sequence::
Day 1 (Mon, after the pressure exposure): Acclimation to testing room and apparatus (5 min/mouse).
Day 2 (Tues): Mouse will be placed in the room with rotarod apparatus. The rotarod will be set at a start speed of 4 rpm, acceleration rate 20 rpm/min. The mouse will be held by the tail, and then placed on the rotating rod, facing it away from the directions of rotating so it has to walk forward to remain upright. Acceleration will start at 10 sec after placing the mouse on the device. The speed at with the mouse falls will be recorded. If it falls off before 10 sec, the time of fall will be recorded, and will try again, up to three times in total. To allow recovery of muscular strength and a return to normal levels of arousal, mice will be rested by a return to the home cage after each motor test. Recording the speed at the first fall after the 10 sec point. The mean speed at fall is the datum. Bubble wrap is placed on the table under the rod to reduce chance of injury on displacement from rotating rod. The wrap is replaced after cleaning with soap water/ towel dried if soiled.
Days 3-5: Testing will be repeated daily to evaluate persistence of deficits (recovery has been described in some studies).

What is the longest that any one animal will be involved in an experiment? **5** days

List each experimental and control group, including the group size for each group (n=?).
Each group will involve 8 male and 8 female mice in 12 groups: Control or decompression, plus injection with PBS, or agents found effective in studies described above. We hypothesize that 3 agents will be found effective.

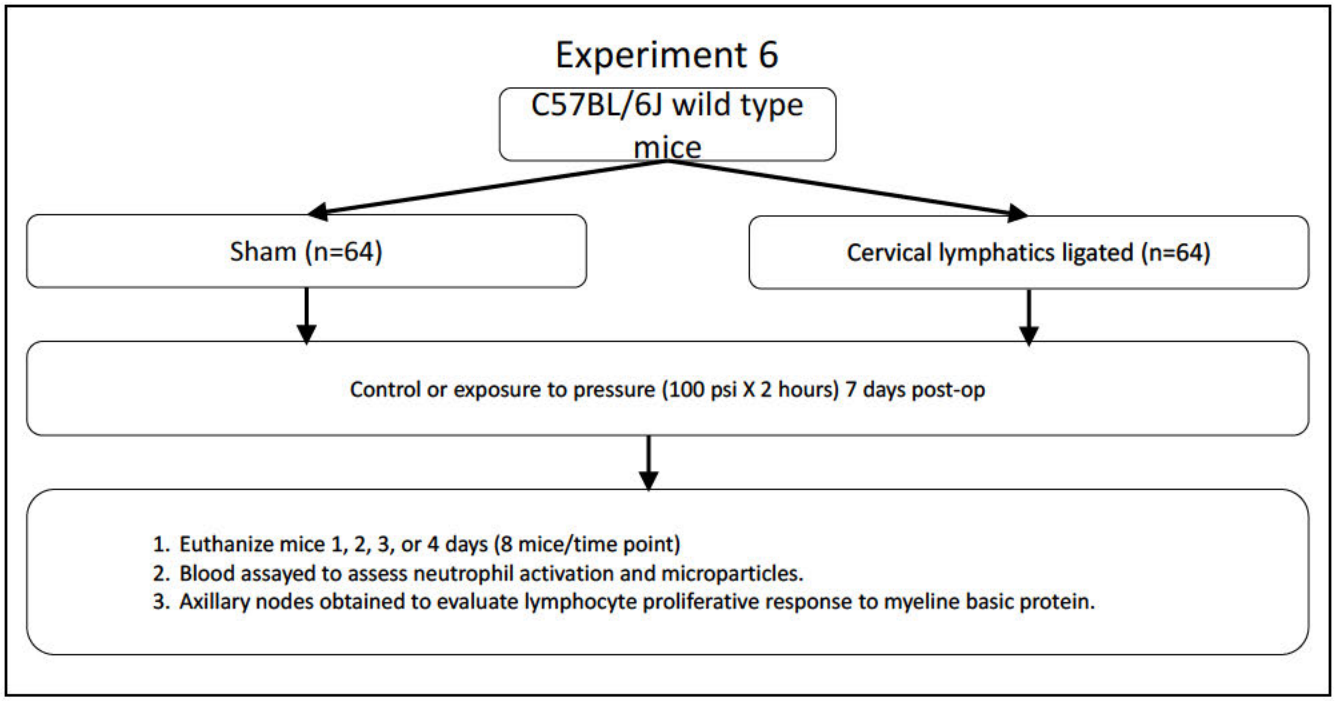
Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data (*e.g., power analysis, reports in the literature, previous experience, etc.*). Prior studies looking at microparticle responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. With functional testing, we anticipate there will be greater variability versus the biochemical studies and there is also greater likelihood that sex will influence the results. Hence, we plan to study 8 male and 8 female per group. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

Indicate whether the experiment must be repeated and justify the number of repetitions.
No repetitions are necessary.

Indicate the species to be used, and how many will be used *(e.g., X # mice per experiment x X # strains of mice).* 16 mice/group X 3 groups X 3 tests = 144 mice

Experiment 6 – Direct assessment of glymphatic outflow causing systemic immune response.

Briefly describe the objective of this experiment. We hypothesize that CNS-generated MPs that first lodge in cervical lymph nodes are responsible for establishing the decompression-induced inflammatory cycle by gaining access to the blood stream where they activate blood-borne neutrophils that then impinge on the blood brain barrier (likely via CD36 receptor). This is supported by preliminary data. The glymphatic system represents a



likely path for CNS-derived MPs to gain access to the peripheral circulation. Activated microglia can trigger neurotoxic T-cell activation via IL-1β (possibly via MPs) and reciprocally, stimulated T-cells activate microglia, and MBP tolerance induction diminishes T-cell mediated microglial activation. Studies show brain protein efflux via glymphatics following trauma, and obstructing lymphatic outflow can abrogate CNS autoimmune responses.

Describe what will happen to the animals from start to finish. We will directly evaluate the role of lymphatic efflux using methods described by van Zwam, *et al.* in their studies of experimental allergic encephalomyelitis (J Pathol 217: 543-551, 2009) [the point being that these procedures are well described in the literature]. The superficial and deep cervical lymph nodes are readily identified. Channels will be ligated, and nodes removed in parallel with sham-operated controls. One week later when mice have recovered from surgery, they will be exposed to pressure (100 psi X 2 hours). We hypothesize that after lymphadenectomy (versus sham) we will see decompression-induced astrocyte activation (*e.g.* NF-kB changes) but there will be no elevations of blood MPs that we believe cause progression of the decompression insult (*e.g.* neutrophil-derived MPs, loss of aquaporin 4 and other manifestations of neuroinflammation). We will also harvest lymphocytes from axillary nodes to assess proliferative response ("priming") to myelin basic protein following our published methods (see (b) (6) [redacted]

What is the longest that any one animal will be involved in an experiment? 12 days

List each experimental and control group, including the group size for each group (n=?). Sham and lymphadenectomy groups (8 mice/group), control (air exposure only) or exposed to high pressure, euthanize mice at day 1,2,3 or 4 post-exposure.

Discuss what criteria were used to determine that the group size(s) proposed utilizes the minimal number of animals to generate statistically significant data. Our prior studies looking at MPs responses have required replicates of up to 8 per group to overcome variability and achieve statistical significance. Based on ANOVA we anticipated this number will achieve a 95 % confidence limit and significance level of $p<0.05$.

Indicate whether the experiment must be repeated and justify the number of repetitions. No repeats.

Indicate the species to be used, and how many will be used. 4 groups x 8 mice/group, 4 time points = 128 mice

4. **Animal Reuse – Will any of the proposed experiments utilize animals that have already undergone experimental procedures in this animal usage protocol <u>or</u> in a separate animal use protocol**? ☐ YES ☒ NO

<u>Detailed Descriptions of Animal Procedures</u>: *Identify <u>EACH</u> procedure to be performed on an animal. List as a subheading and provide a detailed description of how the work will be performed. <u>Please list procedures chronologically</u>. The following items must be addressed.*

<u>Animal Transport:</u> Mice in the vivarium will be transported according to procedures outlined in Veterinary Resources SOP #608.

<u>Exposure of mice in the hyperbaric chamber:</u> Typically, two mice but never more than four are placed in a steel chamber manufactured by Bethlehem Steel Corp. with a volume of ~ 2154 cm^3 (slightly smaller volume than a shoe box mouse cage) that is pressurized with air to up to 100 psi pressure. Chamber pressure is monitored by a sensitive gauge hydrostatically calibrated by the manufacturer. A lab worker is constantly monitoring the chamber pressure and intermittently looks into the chamber using the Plexiglas view port to monitor mouse activity. There is no national certification for animal use hyperbaric chambers but ours has been inspected by the Office of Naval Research grant program officer (who funds the study). Animals move around freely inside the chamber for the 2 hour exposure. Water is provided by a pressure-vented bottle. Air is flowed through the chamber at a rate of 5 liters/minute for the duration of the exposure to maintain a normal atmosphere without carbon dioxide build-up. Pressurization occurs slowly over a span of approximately 10 minutes (30 feet of sea water/5 minutes, same pressurization rate often used by human SCUBA divers) while mice are continually observed through a Plexiglas porthole. There is no notable discomfort experienced by the mice so long as pressurization does not occur at a rate that exceeds the ability of the animal to equalize pressure across their middle ear. Inadequate equalization is reflected by mice shaking their heads and exhibit excess activity during pressurization. If the chamber operator observes this behavior, pressurization is stopped for several minutes until the mouse appears calm and then pressurization is resumed as a slightly slower rate. Although we have never observed a mouse being intolerant to pressure, if a mouse appeared agitated with any pressurization this would suggest there is a mechanical obstruction rendering the mouse unable to equalize middle ear pressure. If that occurred, the mouse would be removed from the chamber and euthanized **.** Depressurization occurs at the same rate as pressurization (over ~ 10 minutes). This model has been used continuously for over 15 years and no mice have ever manifested evidence of decompression sickness such as unconsciousness, paralysis, dyspnea, change in behavior such as hyper- or hypo-activity, or hemoptysis. After each use, the pressure chamber is disinfected with 70% isopropyl alcohol and air dried.

<u>MRI studies:</u> After pressure exposures and placement of the tail vein catheter be done in the PI's lab, mice will be transported to [redacted]. The animal will be anesthetized using isoflurane at a dose range of >3 - 4.5% for induction and >1 - 3% for maintenance via calibrated, precision vaporizer with scavenger system. The animal will then be placed prone in an animal holder with a nose cone and a bit bar. The surface RF array coil will be positioned over the head and the animal's eyes will be treated with a petrolatum based ophthalmic lubricant without antibiotics to prevent corneal desiccation. Typically, the scan is stopped at intervals of ~20 minutes when signal is checked and at these times the corneal desiccant will be re-applied, and level of anesthesia checked and recorded in a study log. The animal holder that is cleaned between uses with soap and water and manually scrubbed will be moved to the center of the magnet and the isoflurane level will be changed to ~ 3 %. The level of isoflurane will be further adjusted based on the respiration rate changes of the animal for the remainder of the experiment. A MR compatible small-animal monitoring and gating system (SA Instruments, Inc., New York, USA) will be used to monitor the animal respiration rate and body temperature. The animal body temperature will be maintained at 35-37°C using a warm water circulation blanket (scientific grade) with a disposable chux pad between mouse and blanket to prevent burns. After moving the imaging bed into the magnet, baseline calibrations will be done followed by injection of pharmaceutical-grade gadodiamide (Gd, 0.12 g/kg in a volume of 0.1 ml) via an IV catheter in the tail vein. When the injection finishes, image acquisitions will follow. After the whole data acquisition, which will be take not more than 2 hours, the animal will be moved out from the magnet and the isoflurane will be stopped. The catheter will

be removed, skin swabbed with 70% alcohol. The injection site will be observed for 5-10 minutes while the mouse is awakening from anesthesia to be assured there is no hemorrhage (if hemorrhage occurred, the site would be held gently with gauze to stop the bleeding). Animals will be provided thermal support during anesthesia recovery and then taken to the PI's lab for euthanasia.

Intra-cisternal injections for fluorescent imaging: This is a non-survival surgical procedure with a maximum duration of 2 hours. Mice will be placed in a precision vaporizer with charcoal scavenging waste system will be used. Mice will be placed in an induction chamber and using 100% oxygen as the delivery gas, anesthesia will be established with an induction dose of >3 to 4.5% isoflurane. Once the animal is recumbent the chamber will be flushed with oxygen and the animal placed in a stereotactic apparatus that was pre-washed with soap, water and then dried. The apparatus has a heating plate (scientific grade) to maintain mouse temperature. A lubricated tip pf a rectal probe is inserted to monitor temperature and maintain at 36oC during the procedure. Ocular lubricant is applied and re-applied every 20 minutes. A lack of toe pinch response is verified prior to proceeding. The scalp will be prepared by clipping with a #40 blade or smaller. The site will be prepped with a surgical scrub soap rinsed with 70% alcohol swabbing (alternating washes repeated for a total of 3 times) and then after a ~0.7 cm longitudinal scalp incision with clean, soap-washed/rinsed instruments and scalp skin is gently retracted using a sterile cotton swab to expose the skull. Using predefined stereotaxic coordinates at 1 mm posterior and 3.5 mm lateral to the bregma the skull is marked as the intended injection site. A dental drill is used to place a burr hole piercing to the inner cortical layer of the skull at these coordinates using a 0.45 mm clean soap-washed/rinsed burr drill bit using refined circular movements. The drill is then removed and a syringe injector in the stereotactic apparatus is used to insert a 33-gauge needle to 1.5 mm below the surface of the brain. At that point 0.5 µl fluorescent tracer is injected. The mouse is removed from the apparatus, kept under isoflurane anesthesia and scalp skin closed over the skull with a single 4-0 nylon suture. At times from 15 to 120 minutes mice will be euthanized by exsanguination. During this time anesthesia checks based on toe pinch are performed at 15-minute intervals. At the designated time euthanasia is carried out. After blood is obtained, the right heart will be opened, a cannula placed in the left ventricle and mouse perfused first with saline until clear effluent returns to the right heart blood followed by gravity-perfusion for 15 minutes with 10% neutral buffered formalin (NBF). Mice will be constantly observed through this procedure. Throughout the observation period mice will be kept on a warming pad (scientific grade) to maintain thermal support.

Blood collections: All blood collection will be performed under anesthesia at time of euthanasia and at no other times.

Routes of Administration:
Intravenous IV (tail vein) – For injections the mouse will be placed in a plastic restrainer that allows access to the tail vein. The Tail vein injection site is wiped with 70% alcohol and a sterile 29G (purchased from SAI Infusion Technologies, Inc.) catheter inserted into the tail vein for IV administration.

SC – Mice will be restrained by scruffing, with the head slightly tilted down. The injection site will be prepped with 70% alcohol. The skin/fur on the back of the neck will be pulled back (i.e,. "tented) to allow insertion of the needle subcutaneously and the contents of the syringe delivered under the skin. The injection volume will not exceed 0.6 ml and needle gauge will be 26 G or smaller.

IP - Mice will be placed in a plastic restraint device that allows free access to the abdomen. After the fur and abdominal wall are wiped with 70% alcohol, a sterile 27 gauge needle will be inserted along the lateral aspect of the mid-abdomen for agent injection. The total volume of solution will not exceed 0.2 ml.

Functional testing protocols:

Novel Object Recognition Test

Normal rodents naturally examine novel objects longer than familiar ones. This natural tendency permits assessment of perirhinal cortex dependent memory without stressful conditions, or high task demands. Memory of familiar objects can be tested at both short and long intervals, to detect deficits in short and long-term memory. Briefly, mice are placed in an arena housing 2 identical objects that are cleaned between uses with soap and water, manually scrubbed and dried prior to repeat usage. Mice are permitted to explore freely. This and subsequent sessions are video recorded for offline analysis. After a retention interval, the mouse is returned to the arena and one of the objects is replaced with a new one. It is expected that mice with an intact memory for the familiar object will spend more time examining the novel object.

For the short (1 hr retention) test, each mouse is allowed 5 minutes to explore 2 identical objects. They will then be removed to the holding cage for a 1 hour 'retention interval' and then replaced in the testing arena and allowed 5 minutes exploration on 1 old and 1 new object. The animals will be monitored continuously during this activity and total testing time for each mouse is estimated to be 1 hour and 15 minutes to allow for lag-times with animal transfer. Mice will be returned to the vivarium at the test session.

For the 'long-term' (24 hr or more retention) test, after taken from the vivarium and returned to the testing site each mouse will be placed in the testing arena after the 24 hour 'retention interval' and allowed 5 minute exploration on the 1 old and 1 new object. The animals will be monitored continuously during this activity and total testing time for each mouse is estimated to be 15 minutes to allow for lag-times with animal transfer.

Beam Walk Test
This study will assess motor and balance functions, as are often impaired in DCS. Mice will be acclimatized to the apparatus after the pressure exposure and daily testing conducted starting 24 hours later. Fine motor coordination will be evaluated by assessing distance and time that mice can ambulate across a 1 m beam (3cm, 2cm, 1cm width). The area beneath the beam has a soft padded surface to cushion any mice that fall. The ability of the mouse to walk will be assessed, measuring distance and time. After each use, the beam and padding are disinfected with 70% isopropyl alcohol and air dried.

Accelerating rotarod Test
Mice will be acclimatized to the apparatus after the pressure exposure and daily testing conducted starting 24 hours later. The mouse is placed in the room with rotarod apparatus and the rotarod set at a start speed of 4 rpm. The mouse is held by the tail and placed on the rotating rod, facing it away from the directions of rotating so it has to walk forward to remain upright. After ambulation starts, acceleration starts at 10 sec after placing the mouse on the device. The speed at with the mouse falls will be recorded. If it falls off before 10 sec, the time of fall will be recorded, and will try again, up to three times in total. To allow recovery of muscular strength and a return to normal levels of arousal, mice will be rested by a return to the home cage after each motor test. Recording the speed at the first fall after the 10 sec point. The mean speed at fall is the datum. Bubble wrap is placed on the table under the rod to reduce chance of injury on displacement from rotating rod. The wrap is replaced after cleaning with soap water/ towel dried if soiled. Testing will be repeated daily to evaluate persistence of deficits (recovery has been described in some studies). After each use, the beam and padding are disinfected with 70% isopropyl alcohol and air dried.

Video recording of cognitive tasks: For each of the tasks described above, the mice will be recorded on video to aid with recording of time to achieve each of the tasks. We will comply with all UMB SOM IACUC guidelines on image recording. Specifically, we do not anticipate including image / recording in publications or exhibitions. However, if ever we do think this is a reasonable activity, we will obtain IACUC approval of the image / recording prior to use. Further, with capturing all image / recording, we will be careful to limit the visibility of the animal facility and research staff. Moreover, all images / recordings will be removed from the recording device and stored on a secured device.

Cervical node MPs procurement: This is a non-survival surgical procedure in which mice are anesthetized (Ketamine/xylazine) and the neck and chest are fully clipped and then swabbed with 8-12% solution of either betadine or chlorhexidine sudsing surgical scrub applied with clean cotton gauze, then rinsed with 70% alcohol on gauze and both steps repeated two more times. The last application of the alcohol is allowed to air dry. Once a level of surgical anesthesia is reached, the neck and thorax are aseptically opened and exsanguination performed followed node harvesting. The neck is dissected, cervical lymph nodes identified and harvested for *ex vivo* mincing and microparticle purification. This is achieved by centrifugation in sterile tubes for injections into the tail vein of recipient mice (as described above under IV tail vein injection). This procedure is performed in the PI's lab

Survival surgery – ligation of cervical lymphatics: Prior to pressure exposure, mice will have deep cervical lymphatic chains ligated to prevent release of CNS-derived MPs from gaining access to the blood stream. Isoflurane anesthesia will be induced via induction chamber and continued post induction via a face mask. Post skin prep, surgery will be initiated as described in detail below and blood flow will be monitored (to be assured we do not disturb flow during the surgery). Cerebral blood flow will be monitored by Laser Doppler Flowmetry (LDF) (Moor Instruments, Inc.

Wilmington, DE) during the procedure. Servo controlled thermal support is provided to maintain steady by temperature. Sham animals will undergo the same procedure except that lymphatic ligation will not be done. To mitigate the pain and distress, we will use a local anesthetic (bupivacaine) at surgery to reduce immediate post op pain and will euthanize mice early if exhibiting signs stated in the 'Alternative Endpoints' section below.

Surgical Area Prep: In order to maintain aseptic conditions, the surgical desk and other accessories will be free of clutter, cleaned with 10 % dilution of household bleach, wiped dry with a clean towel, and covered with a clean drape. Washed and cleaned instruments will be autoclaved at 121 º C for 20 min.

Surgeon prep: The surgeon will don a facemask, wash hands with antiseptic soap, dry hands on clean paper towels and don a surgical gown, and then sterile gloves. Gloves will be changed between animals or if grossly soiled or break in aseptic technique occurs (e.g., glove accidentally touches non-sterile objects).

Instrument Prep: For first use on any day all instruments will be autoclaved. When conducting multiple surgeries on the same day, between use on animals, the instrument tips will be rinsed clean of any organic debris and the distal 1/3 of the instruments will be re-sterilized in a bead sterilizer and placed on sterile drape or roll of sterile cotton gauze to air cool a minimum of 5-10 minutes until next use.

Anesthesia: Mice are weighed, and weights recorded on their anesthesia records. A precision vaporizer with charcoal scavenging waste system will be used to delivery isoflurane. Mice will be placed in an induction chamber and using 100% oxygen as the delivery gas, anesthesia will be established with an induction dose of >3 to 4.5% isoflurane. Once the animal is recumbent and respiration noted to slow, the chamber will be flushed with oxygen and the animal moved to the to an area a few feet from the 'surgery table. Anesthesia will be maintained via nose cone using Isoflurane at >1 - 3 % in 100 % O_2 and monitored continuously during and after the procedure, as described below. In any rare cases of a non-functioning isoflurane system, Ketamine (80-150mg/kg)/Xylazine (8-15mg/kg) will be administered intraperitoneally post manual scruffing of mice by grasping them at the nape of the neck. Injection will be done at a mid-abdomen injection site after prep (70 % alcohol on clean cotton gauze,) using a 25 G or smaller bore needle to induce for surgical prep and this surgery and surgical plane of anesthesia confirmed as described in above. If top off is needed at around 25 minutes post-induction, ½ the initial dose of ketamine will be administered, and if at 45-50 minutes if top off is needed then ½ doses of ketamine and xylazine will be administered.

Surgical Site Prep: In an area located approximately 1-2 feet from the surgery table, fur is removed from both sides of the neck of the anesthetized mouse by using electric clippers with a # 40-50 clipper blade, fur is removed to provide 1 cm margins around the planned incision lines. The clipping margins will be from brow to back of skull, and from ventral to dorsal midline. The clipped areas will be wiped with a gauze wet with 70% ethanol to remove any loose fur. Post removal of lose fur, animals are moved to the surgery table.

Thermal Support: During the ~ 60 min surgical procedure, animals will be maintained at a temperature of 37.0±0.5 degree Celsius with a homoeothermic-heating pad (Physio Suite Mouse STAT Right Temp, Kent Scientific Co., Torrington, CT). Note that a disposable chux pad will be placed between the mouse and the heating pad to prevent burns.

Ocular Protectant: Protective ocular ointment (Para-lube or Lacri-lube) is placed in both eyes to reduce chances of corneal desiccation. Application will be repeated every 20-30 minutes post induction of anesthesia.

Aseptic Prep: The Clipped skin area is prepped with and 8-12% solution of either betadine or chlorhexidine sudsing surgical scrub applied with clean cotton gauze, then rinsed with 70% alcohol on gauze and both steps repeated two more times. The last application of the alcohol is allowed to air dry.

Surgical Procedure:

1) Depth of the anesthesia will be confirmed by a lack of corneal reflex and/or no response to deep tail or foot pinch. Anesthetic depth will be re-assessed at least every 10-15 minutes throughout the surgery and noted on anesthesia record (adequate, increased, decreased etc).

2) Local analgesia will be provided by injecting approximately 0.2 ml of bupivacaine (0.25% - 0.5%, diluted 1:1 with saline, max 6 mg/kg) subcutaneously in the incision sites with a 25 G or smaller bore needle, which will provide local anesthesia for approximately 6-8 hrs.

3) After skin prep a small incision (3-4 mm) will be made with sterilized scissors between the ear and the eye.
4) The muscles under the skin will be separated with the same scissors to expose the temporal bone for inserting a needle laser Doppler probe (diam. 2 mm, or 20G) which will allow monitoring of cerebral blood flow throughout the procedure. Note that the Laser Doppler is an instrument dedicated for animal use.
5) The Doppler probe will be cleaned thoroughly with soap and water then rinsed with 70% ethanol and cold sterilized in Cidex overnight each day. It is rinsed with sterile water to remove Cidex prior to placement in the animal.
6) The probe is inserted at the bone above the middle cerebral artery territory and signal monitored throughout the surgical procedure.
7) Bilateral ventral cervical incisions, approximately 1-1.5 cm long will be made and the major vessels in the carotid sheath will be identified.
8) Lymphatic vessels and nodes are found in this region and easily identified. Nodes will be removed, and vessels ligated with a sterile 7-0 nylon monofilament surgical suture.
9) The ventral cervical incisions and incision for the LDF probe will be closed with FDA approved surgical glue (Nexaban, Vet Bond) cyanoacrylate tissue adhesive applied with TB syringe and 25 G needle. If bulging at the incision is noted at this point, 5-0 nylon simple sutures will be added.
10) The entire time for surgery will not exceed 60 minutes under any circumstances. After incision closure, animals will be placed in a recovery cage for the recovery period and the body temperature will be maintained at 37.0±0.5°C through use of Stryker T/Pump and warm water recirculating pad. The heating pad will be only be placed under half of the cage bottom to allow the animal to move away from the heat source if desired. After surgical completion mice will receive sub-cutaneous buprenorphine that will be re-administered twice daily for the one-week recovery period.
11) Once the animals are ambulatory, they will be returned to their home cages that have a supplemental heat source on one side and clean dry bedding. Once fully ambulatory with no signs of abnormal behavior, the supplemental heat source will be removed, and the mouse returned to the Animal Facility.
12) The laboratory staff will monitor the mice twice daily for the rest of the week. The animals will be visual inspected daily for any clinical signs, including on weekends. At any sign of redness or edema, a comparative medicine veterinarian will be contacted to evaluate the animal and make further recommendations. If the animal is exhibiting signs of being in pain (reluctance to move, eat or drink and vocalizations with handling), a veterinarian will be consulted. If the animal is too sick to recover, it will be immediately euthanized.
13) A green "Small Animal Surgery/Procedure" card obtained from VR animal care staff will be placed on the cage of each animal undergoing surgery. The front of the card will indicate date and type of procedure, as well as the contact person with both cell and lab numbers.
14) At 1 week post-op well appearing mice will undergo pressure exposure protocol and then studied at times up to 4 days later. If findings from Experiments 1-7 suggest that observation periods need to be longer than 4 days we will submit an addendum to IACUC to modify the procedure.

Exogenous Substances: *(any substance administered to a live animal)*

Substance	**Dose Range** *(mg/kg)*	**Route**	**pharmaceutical grade*** *(yes / no)*	**Chemical Hazard?** *(No, CL1, CL2)*	**Purpose** *(anesthetic, analgesic, paralytic, experimental, etc.)*
Gadodiamide	0.12 mg/kg	IV	Yes	No	**Gadolinium contrast agent**
Gelsolin	1 ug/kg	IV	Yes	No	Experimental
PEG-Telomer B	0.002 mg/kg	IV	Yes	No	Experimental
Anakinra	100 mg/kg	IP	Yes	No	Experimental
JSH (4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine)	3 mg/kg	IP	Yes	No	Experimental
KYC (acetyl-lysyltyrosylcysteine)	0.3 mg/kg	IP	Yes	No	Experimental
PBS saline	10 ml/kg or 10ml	IV	Yes	No	Solvent, perfusion
isoflurane	>3 - 4.5% for ind and >1 - 3% for maintenance; > 4.5% for euthanasia.	Inhalation	Yes	No	Anesthesia – delivered by calibrated, precision vaporizer with scavenger system

Ocular protectant (lacrilube)	n/a	Each eye	yes	No	Eye protection
Fluorescent cadaverine	0.1 mg/kg	Intra-cisternal	No –but sterile and filtered	No	Brain glymphatic imaging
Fluorescent ovalbumin	0.1 mg/kg	Intra-cisternal	No –but sterile and filtered	No	Brain glymphatic imaging
ketamine	80-150 mg/kg	IP	Yes	No	Anesthetic
xylazine	8-15 mg/kg	IP	Yes	No	Anesthetic
Buprenorphine	0.05-0.1 mg/kg	SQ	Yes	No	Post-op analgesic
bupivicaine	0.2 ml (0.25% - 0.5%, diluted 1:1 with saline, max 6 mg/kg)	SC	Yes	No	Local analgesia
*Scientifically justify use of non-pharmaceutical grade compounds: Non-pharmaceutical grade agents will be used only when pharmaceutical grade agents are not available.					
☒ *I confirm any non-pharmaceutical grade compounds will be reconstituted and stored per the *IACUC Guidelines on the use of Non-pharmaceutical Grade Compounds in Animal Use Protocols*.					

CL1 – Chemical Level 1; CL2 – Chemical Level 2; Refer to EHS Hazardous Chemical Use in Animal Research website for guidance.

Identification of Animals: *Please provide details on the method of identification (e.g., ear tagging, tattooing, etc.) used for all animals in the study and the age of animals when procedures are performed.*

Mice do not have to be tagged or tattooed. We will use ~20 gram, 8 week old mice. Mice will be identified at the cage level by **marking the ears or tail with a color using an ink pen**.

General Monitoring: *Please provide details to the general monitoring of animals throughout the course of these experiments. Define parameters to be monitored, frequency of monitoring (at least 3 times a week), and identify the individual(s) responsible for monitoring.*

Mice are under observation daily Monday through Friday and provided with extra food and water over weekends. All animals will have free mobility and access to food and water. During the study, we will monitor for general signs of animal distress. If mice were found to be behaving as if in pain based on abnormal behavior (hyperactive, or not moving at all), exhibiting piloerection, or porphyrin deposits from eyes they would be immediately euthanized.

Alternative Endpoints: *Please specify criteria to be used to determine whether early euthanasia (prior to the experimental endpoint) is warranted. Please refer to the Endpoint Guidelines available on the OAWA website and include only those that are applicable to the proposed research.*

There are no specific manifestations to adverse effects from decompression, or other manipulations planned in this project. That is, one would look for general signs of animal distress. If mice were found to be behaving as if in pain based on abnormal behavior (hyperactive, or not moving at all), exhibiting piloerection, or porphyrin deposits from eyes they would be immediately euthanized (ketamine/xylazine and then exsanguination). None of the agents planned for use should cause animals to lose weight or suffer any other sort of stress in the short time they will be under observation until euthanasia.

Regarding animals subjected to survival surgery, if mice appear moribund as defined below they will be euthanized.

(i) 15 % weight loss over 24 hours: The percentage of weight loss = (Pre surgery weight minus weight on any day) divided by pre-surgery weight x 100. For animals after surgery, body weight will be assessed every other day starting on day 1 post op.
(ii) Inability to rise or ambulate.
(iii) Inability to eat/ drink for 24 hrs.
(iv) Recumbent mice that are unresponsive to gentile finger prodding.
(v) Evidence of CNS coma signs (*e.g.* no pupillary reflex).
(vi) Diarrhea for more than 3 days.
(vii) Self-induced trauma.

(viii) Incision line infection (red/ swelling, discharge or dehiscence).
(ix) Bleeding from any orifice.
Animals with any one of these conditions will be considered as moribund and euthanized by ketamine/xylazine followed by exsanguination for tissues collection/ analysis.

Research Personnel Qualifications, Experience or Training: *Please provide the following information for each person working with live animals under this protocol. List the PI information first, then any Co-PIs, etc.*

Name, Email & Phone #	**Indicate role in project and list procedures he/she will be performing.**	**Indicate years' experience working with the *species* of animals proposed for use in this protocol.**	**Indicate years' experience performing the *proposed procedures in the species* utilized in this protocol.**	**If the individual is not trained in either the procedures or species (*or both*), please indicate how he/she will be trained.**
Stephen R. Thom (b) (6) [redacted]	Principal Investigator; IV & IP pharmaceutical administration, cervical lymph node ligation/survival surgery, behavioral protocol, **high pressure exposures/decompression**, mouse euthanasia	33	26	Trained person
(b) (6) [redacted]	Lab manager, IV & IP pharmaceutical administration, **high pressure exposures/decompression,** behavioral protocol, mouse euthanasia	27	16	Trained person
(b) (6) [redacted]	Research Associate, IV & IP pharmaceutical administration, cervical lymph node ligation/survival surgery, behavioral protocol, **high pressure exposures/decompression,** mouse euthanasia, **colony maintenance and lab animal monitoring.**	7	3	Trained person
(b) (6) [redacted]	Post-doctoral fellow, IV & IP pharmaceutical administration, cervical lymph node ligation/survival surgery, behavioral protocol, **high pressure exposures/decompression**, mouse euthanasia	3	2 months	Trained person

***Please add rows to this table by placing cursor at end of last row and clicking return.*

☒ As PI, I confirm that no minors will be working with live vertebrate animals per UMB Policy VI-99.01(A).

☒ As PI, I acknowledge that a COI Exemption must be filed with the UMB Office of Accountability & Compliance should my spouse or a family member work under this animal use protocol per UMB Policy.

BUMED Animal Use Appendix for Research Involving Animals
ABBREVIATED VERSION

*****NOTE: BUMED will ONLY review protocols that have been approved by your IACUC.*****
****Animal work MAY NOT be initiated until the awardee receives BUMED approval.****
Animal work initiated without BUMED approval is noncompliant and may not be funded.

Institutions using DoD funds to support the use of animals in research, product development, testing and education projects must provide electronic copies of the following documents to the Department of the Navy (DON) Bureau of Medicine and Surgery (BUMED) Veterinary Affairs Office for review and approval prior to initiation:

a. A copy of their **IACUC-approved institutional protocol(s)** (BUMED will ONLY review **approved** protocols) and documentation of IACUC approval

b. A copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by BUMED **PRIOR** to implementation)

c. A completed Appendix for each IACUC-approved protocol.

This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs.

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DoD regulations. The DoD policies and requirements for the use of animals in DoD-supported research, development, testing and evaluation are described in DoD Instruction 3216.01, dated September 13, 2010 and SECNAVINST 3900.38C, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to BUMED Veterinary Affairs:

Phone: [redacted]
Email: DON – VRPP, [redacted]
Mail: Department of the Navy Bureau of Medicine and Surgery
ATTN: Director for Veterinary Affairs
Bldg 1564 Room 122
1564 Freedman Drive
Fort Detrick, MD 21702

~~~~~~

Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text. Submit electronic copies of the appendix only; please do not submit printed copies to BUMED. Any section of the Appendix that is not applicable to your proposal, e.g., no surgery or no prolonged restraint, should be marked "**No**" or "**N/A**". There are no space limitations for the responses.

It is essential that only animal studies or procedures documented in an IACUC–approved protocol or amendment be performed at your facility. BUMED will collect animal usage information for end-of-year DoD animal use reports separate from the grant annual progress reporting requirement throughout the life of your award. For this reason, Principal Investigators or other delegated research personnel should keep accurate records and be able to provide an audit trail of all animal use that correlates to their approved protocol.
~~~~~~

[x] Yes [ ] No All animal studies described in the attached IACUC-approved protocol are funded by this DOD award/contract. If no, STOP! You must use the **full version** of the Animal Use Appendix.

1. Administrative Data: (Provide Attending Vet, IACUC, and Research Office info for the work site.)

DoD/Contract PI Name: Peter Lindholm

Grant/Contract PI Email: [redacted] **Phone:** [redacted]

Protocol PI Name: Peter Lindholm

Protocol PI Email: [redacted] **Phone:** [redacted]

Animal Research Site (RS): University of California San Diego

RS Attending Veterinarian: [redacted]

Attending Vet Email: [redacted] **Phone:** [redacted]

RS IACUC Point of Contact (POC): [redacted]

IACUC POC Email: [redacted] **Phone:** [redacted]

RS Grants Office Point of Contact (POC): [redacted]

Grants Office POC Email: [redacted] **Phone:** [redacted]

Animal Protocol Title: Nitrogen gas uptake and excretion during compression/decompression to simulate diving

2. Total Number of Animals Used (by Species) and USDA Pain/Distress Category :

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Rattus Norvegicus	D	58

USDA Pain/Distress Category Definitions:

- ***Column B:*** *Animals being bred, and animals being held for use in research, testing, teaching, experiments or surgery but not yet used for those purposes.*
- ***Column C:*** *List the number of animals that will experience no more than slight or momentary pain or distress as a result of experimental manipulations or procedures on this protocol.*
- ***Column D:*** *List the number of animals that will potentially experience more than momentary or slight pain or distress that **WILL** be alleviated through the use of anesthetics and/or analgesics.*

- ***Column E:*** *List the number of animals that will experience more than momentary or slight pain or distress that* ***WILL NOT*** *be alleviated or relieved with anesthetics or analgesics.* **If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:**. .

3. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals. Note the DoD-specific database requirements in subparagraph a.

a. Literature Source(s) Searched:

http://www.ntis.gov/products/fedrip.aspxhttp://projectreporter.nih.gov/reporter.cfm

[ ] Federal Research in Progress (FEDRIP) http://www.ntis.gov/products/fedrip.aspx

or

[x] Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER) http://projectreporter.nih.gov/reporter.cfm

b. Date of Search: March 11, 2020

c. Years Covered By Search: 1995-2020

d. Key Words and/or Search Strategy: 13N2, Decompression, Nitrogen

e. Results of Search: nothing relevant.

******Questions 4-10 refer to the Research Site and Protocol Principal Investigator.******

Information and/or documents required in questions 4-8 should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to BUMED by the Protocol Principal Investigator (PI) or institution staffs. If the Protocol PI prefers that BUMED staff contact the institution to obtain this information and/or documents, the PI must specifically request this action in writing. The Protocol PI should contact BUMED (see contact information on cover page) to arrange for submission of this written request.

4. Institutional Animal Care and Use Committee(s) (IACUC) Approval(s):

Institutional Animal Use Protocol Number:	S19154		
IACUC (initial) approval date:	11/26/2019	Protocol expiration (rewrite) date:	11/26/2022

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[x] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[x] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

[x] Yes [ ] No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

[ ] Yes [x] No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

[ ] Yes [x] No Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Peter Lindholm — March 11/2020 (b) (6) [redacted]

(Protocol Principal Investigator Printed Name) — (Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

OXIDATIVE TISSUE DAMAGE AFTER HBO_2 EXPOSURE USING SELECTED FDA-APPROVED ANTIEPILEPTIC DRUGS (AEDS)

(b) (6) [redacted], Heath G. Gasier

The Duke Center for Hyperbaric Medicine and Environmental Physiology
heath.gasier@duke.edu

Background

In extreme HBO_2, the onset of central nervous system oxygen toxicity (CNS-OT) is linked to the development of oxidative lung injury.[1] This results from uncontrolled sympathetic activation causing left ventricular dysfunction that leads to left atrial and pulmonary capillary hypertension, and barotrauma. Strategies that provide protection to the CNS in HBO_2 may also reduce the degree of acute lung injury (ALI). We have previously demonstrated varying degrees of antiseizure protection with antiepileptic drugs (AED's).[2,3] Administration of GABA to rats protects the lung and brain from oxidative tissue damage.[4] Intratracheal or oral GABA administration attenuates ventilator-induced lung injury in rats and pneumonitis and death in mice infected with murine hepatitis virus 1.[5,6] Providing a GABAeric enhancer alone or in combination with another AED should reduce ALI in extreme HBO_2, the focus of this work. In addition, because there is no established biomarker for CNS-OT, we measured serum S100 calcium binding protein B (S100B), an astrocyte protein that leaks into the blood upon brain injury and seizures.[7,8]

Objectives

To determine the efficacy of AEDs on acute lung injury in mice exposed to HBO_2 at 5 ATA for 60 min. The AEDs tested were tiagabine (TGB), a GAT1 inhibitor, and carbamazepine (CBZ), a sodium channel antagonist. A secondary objective was to assess the utility of serum S100B as a biomarker of CNS-OT.

Methods

Male C57BL/6 mice aged 8-10 weeks were exposed to air or HBO_2 at 5 ATA for 30 or 60 min. Thirty min before exposures, mice were injected IP with 0.9% NaCl, TGB or TGB+CBZ. For 30 min HBO_2 exposures, the TGB dose was 4.8 μg/g. For 60 min HBO_2 exposures, the TGB dose was 9.4 μg/g (individual) and 4.8 μg/g when combined with 16.4 μg/g of CBZ. These doses were selected based on the degree of antiseizure efficacy in HBO_2.[3] Following the 30 min exposures, blood was collected and separated for measurement of serum S100B using a colorimetric assay (LSBio). To determine the extent of ALI, we measured the lung wet-to-dry ratios, bronchoalveolar lavage fluid (BALF) protein content (BCA method), lactate dehydrogenase activity (colorimetric assay, Sigma-Aldrich) and myeloperoxidase (MPO) levels (colorimetric assay, Abcam).

Results

(b) (4) [redacted]

(b) (4) [redacted]

In mice exposed to HBO_2 at 5 ATA for 30 min, TGB extended seizure onset by a factor of 2 over vehicle (P=0.0007). In a subset of these animals, we determined that HBO_2 increased serum S100B 128% over air mice independent of treatment (**Fig. 1**). The level of S100B and

seizure latencies were unrelated, suggesting acute brain injury is occurring in extreme HBO_2 even in the absence of seizures.

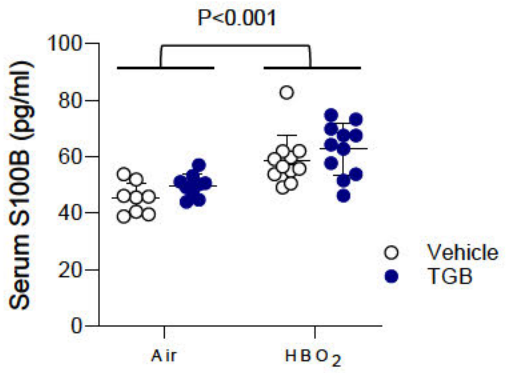


Fig. 1. Serum S100B in mice exposed to air and HBO_2 at 5 ATA for 30 min. Mice were given IP 0.9% NaCl or TGB (4.8 μg/g). Blood was collected immediately after exposures, separated and frozen at -80 °C until measurement. *n* = 8=11/group.

Summary

In mice, a single 60 min exposure to HBO_2 at 5 causes substantial alveolar-capillary barotrauma with a modest increase in lung edema. Combined with a lack of cell damage and inflammation, these data represent the early phase of extreme HBO_2 ALI. Despite early neuroprotection with TGB and TGB+CBZ, the AEDs did not prevent neurogenic-mediated ALI. In mice exposed to HBO_2 at 5 ATA for 30 min, the elevated serum S100B implies the presence of acute brain injury independent of seizure activity. Blood S100B may, therefore, be a useful marker to assess efficacy of CNS-OT mitigation strategies.

Notes:

We thank (b) (6) [redacted] for their technical assistance. I extend my gratitude to (b) (6) [redacted] Professor Emeritus, for his continued support. (b) (6) [redacted] is a critical care pulmonary fellow and involved in several ONR and NAVSEA projects within the Duke Center for Hyperbaric Medicine and Environmental Physiology (CHMEP). His area of interests include acute lung injury and decompression illness. He has been accepted into the Undersea and Hyperbaric Medicine Fellowship at the Duke CHMEP, class of 2022-2023.

References:

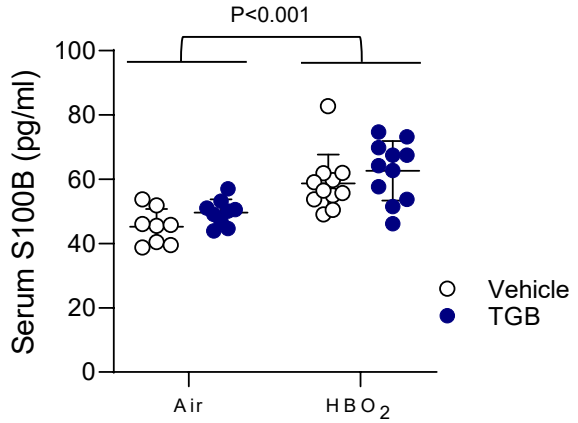
1. Demchenko IT et al. *Am J Physiol Lung Cell Mol Physiol*. 2011;300(1):L102-11.
2. Demchenko IT et al. *Brain Res*. 2017;1657:347-354.
3. Demchenko IT et al. *Neurotox Res*. 2019;36(4):788-795.
4. Wood JD, Stacey NE, Watson WJ. *Can J Physiol Pharmacol*. 1965;43:405-410.
5. Chintagari NR, Liu L. *Crit Care*. 2012;16(2):R55.
6. Tina J, Middleton B, Kaufman DL. *Viruses*. 2021;13(6).
7. Bargerstock E et al. *PLOS One*. 2014;9(7):e101477.
8. Donato R et al. *Curr Mol Med*. 2013;13(1)24-57.

Oxidative Tissue Damage After HBO_2 Exposure Using Selected FDA-Approved Antiepileptic Drugs (AEDs)

Matthew S. Makowski, Hagir S. Suliman, Heath G. Gasier
Duke University Medical Center

Summary: In extreme HBO_2, the onset of central nervous system oxygen toxicity is linked to the development of oxidative lung injury. In male mice pretreated with the antiepileptic drugs (AEDs) tiagabine and carbamazepine before a single 60 min exposure to HBO_2 at 5 ATA, despite early neuroprotection AEDs did not prevent lung edema or alveolar-capillary barotrauma. S100B is a an astrocyte protein that leaks into the blood upon brain injury and seizures. In male mice exposed to a single 30 min exposure to HBO_2 5 ATA, serum S100B increased by 128% over air mice independent of AED treatment or seizure activity.

Naval Application: Increased exposure depth and time for military divers, patients receiving HBO_2, and safe evacuation of crewmembers in a DISSUB.



Serum S100B, a potential marked for assessing acute brain injury in HBO_2. TGB, tiagabine.

Aims: For this project, there were four specific aims. For FY22, our attention as been placed on **Aim 3: Measure inflammatory activity in mouse forebrain and hindbrain, lung parenchyma, and mouse plasma and compare four conditions, air, HBO_2, air-AEDs, HBO_2-AEDs.** We show data for acute lung injury and the blood marker S100B.

Issues: 1) Time delay in PI transfer (Piantadosi to Gasier). 2) Two blood biomarkers were initially proposed, S100B and neuron-specific enolase (NSE). Due to the volume of plasma/serum required to perform these measurements, we selected S100B since it is used as a biomarker for traumatic brain injury.

FY22 Accomplishments, Discoveries, & Inventions

- Completed aim 3.
- Determined that AEDs do not prevent acute lung injury in extreme HBO_2.
- Identified a potential sensitive biomarker of HBO_2 brain injury that is easily measured in the blood with a commercially available assay kit.

FY23 Goals

- Measure gender differences in GABA metabolism and repeated HBO_2 sensitivity.
- Measure time course of repeated HBO_2 oxidative tissue injury.
- Increase sample size of serum S100B and examine if this can be used as a recovery marker.

Abstract

BAA Number: N00014-20-S-B001
Technical Contact (PI): (b) (6)(b) (6)(b) (6)
*Abstract Approved for Public Release

Where is the gas? A new method to study nitrogen during diving and decompression

A **century of study has not been able to identify the causal mechanism of decompression sickness** (DCS). We propose a new quantitative method to study Nitrogen gas (N_2) in mammals. Decompression sickness is currently hypothesized to be caused by bubble formation in tissues. However, due to the lack of direct methods to track tissue gas, **the field has used indirect methods** such as ultrasound of venous gas emboli (VGE) in the heart and mathematical modelling based on empirical testing in human divers. Current methods to predict DCS are based on statistical probability with relative risk factors such as depth and duration. It has not yet been possible to determine the causal mechanisms and explain the intra- and interindividual variability present in DCS, suggesting that **a direct method to map tissue gas distribution is a priority, as it has the potential to refine risk assessment and contribute to optimizing diving profiles.**

We will use a radioactive, positron-emitting isotope of Nitrogen (**$^{13}N_2$**) that can be breathed in a hyperbaric chamber and monitored in time and space using **positron-emission tomography (PET) combined with magnetic resonance imaging (MRI)**. With $^{13}N_2$, we can quantitatively measure the Nitrogen distribution in the body. $^{13}N_2$ has previously been produced and used in normobaric experiments with human volunteers, and a hyperbaric chamber can be constructed to inhale the gas during compression (simulated diving), widening applicability to hyperbaric breathing. We hypothesize that tissue gas bubbles will create bright spots while gas in solution will generate a signal equivalent to that tissue's saturation with Nitrogen. This new method would allow to answer the gas localization question, and address the impact of preconditioning on the amount of venous gas emboli after decompression. This approach will open a window **to study basic mechanisms and impact of interventions** related to DCS in both animals and humans.

Specific aim 1: To develop a method for inhaling $^{13}N_2$ under pressure and imaging gas distribution in a live animal. This will allow direct tracking of Nitrogen gas throughout the body, which is key to mechanistically explain decompression sickness under different conditions. We will **produce and purify the $^{13}N_2$ radioactive gas**, including compression and delivery into a pressure chamber, prior to PET and MR imaging. A chamber able to compress rodents within the PET and MRI will be developed and built. This experiment will establish the **proof of concept** that we can **track gas related to compression-decompression** in a rodent model.

Specific aim 2: Quantitative measurements of Nitrogen gas uptake and distribution during compression and excretion during and after decompression in rodents.

The dive profile (duration, pressure) will alter the tissue uptake of gas. Moreover, it has been shown that preconditioning significantly reduces VGE and reduces mortality by up to 50%. Using exercise as a preconditioning intervention, we will **test the hypothesis that preconditioning reduces the total nitrogen uptake** in rodents. We will also identify changes in **nitrogen storage, spatial distribution, and uptake and excretion temporal dynamics.**

Specific aim 3: Scaling the method to enable large animals. The method developed for rodents will be scaled to enable large animal experiments (e.g. sheep) breathing $^{13}N_2$ under pressure followed by imaging in a PET/MRI. The expected **outcome** is imaging **data** on signal intensity and gas distribution that can be **used to predict risk and optimize doses for planning human experiments**.

The proposed studies will establish a new rat model (and technical preparation for large

animals and humans) that **will enable quantitative measurements of Nitrogen gas in vivo during compression-decompression** thus providing insight into tissue gas distribution and mechanisms of bubble generation during decompression. This technique will provide a valuable new tool for testing and selecting optimal interventions to prevent DCS, and optimize operational diving.

BUMED Animal Use Appendix for Research Involving Animals
ABBREVIATED VERSION

*****NOTE: BUMED will ONLY review protocols that have been approved by your IACUC.*****
****Animal work MAY NOT be initiated until the awardee receives BUMED approval.****
Animal work initiated without BUMED approval is noncompliant and may not be funded.

Institutions using DoD funds to support the use of animals in research, product development, testing and education projects must provide electronic copies of the following documents to the Department of the Navy (DON) Bureau of Medicine and Surgery (BUMED) Veterinary Affairs Office for review and approval prior to initiation:

a. A copy of their **IACUC-approved institutional protocol(s)** (BUMED will ONLY review **approved** protocols) and documentation of IACUC approval

b. A copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by BUMED **PRIOR** to implementation)

c. A completed Appendix for each IACUC-approved protocol.

This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs.

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DoD regulations. The DoD policies and requirements for the use of animals in DoD-supported research, development, testing and evaluation are described in DoD Instruction 3216.01, dated September 13, 2010 and SECNAVINST 3900.38C, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to BUMED Veterinary Affairs:

Phone: (b) (6)(b) (6)(b) (6)(b) (6) [redacted]
Email: DON – VRPP, (b) (6) [redacted]
Mail: Department of the Navy Bureau of Medicine and Surgery
ATTN: Director for Veterinary Affairs
Bldg 1564 Room 122
1564 Freedman Drive
Fort Detrick, MD 21702

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Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text. Submit electronic copies of the appendix only; please do not submit printed copies to BUMED. Any section of the Appendix that is not applicable to your proposal, e.g., no surgery or no prolonged restraint, should be marked "**No**" or "**N/A**". There are no space limitations for the responses.

It is essential that only animal studies or procedures documented in an IACUC–approved protocol or amendment be performed at your facility. BUMED will collect animal usage information for end-of-year DoD animal use reports separate from the grant annual progress reporting requirement throughout the life of your award. For this reason, Principal Investigators or other delegated research personnel should keep accurate records and be able to provide an audit trail of all animal use that correlates to their approved protocol.
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[X] Yes [ ] No All animal studies described in the attached IACUC-approved protocol are funded by this DOD award/contract. If no, STOP ! You must use the **full version** of the Animal Use Appendix.

1. Administrative Data: (Provide Attending Vet, IACUC, and Research Office info for the work site.)

DoD/Contract PI Name: Heath G. Gasier, PhD

Grant/Contract PI Email: (b) (6)(b) (6)(b) (6) **Phone:** (b) (6)

Protocol PI Name: Heath G. Gasier, PhD

Protocol PI Email: (b) (6)(b) (6)(b) (6) **Phone:** (b) (6)

Animal Research Site (RS): Duke University

RS Attending Veterinarian: (b) (6)(b) (6)(b) (6)

Attending Vet Email: (b) (6)(b) (6) **Phone:** (b) (6)

RS IACUC Point of Contact (POC): (b) (6)(b) (6)

IACUC POC Email: (b) (6)(b) (6)(b) (6) **Phone:** (b) (6)

RS Grants Office Point of Contact (POC): (b) (6)(b) (6)

Grants Office POC Email: (b) (6)(b) (6) **Phone:** (b) (6)

Animal Protocol Title: The role of GABA in susceptibility to oxygen toxicity with repeated HBO_2 exposure

2. Total Number of Animals Used (by Species) and USDA Pain/Distress Category :

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Mus musculus	C	96
Mus musculus	D	64
Mus musculus	E	432

USDA Pain/Distress Category Definitions:

- ***Column B:*** *Animals being bred, and animals being held for use in research, testing, teaching, experiments or surgery but not yet used for those purposes.*
- ***Column C:*** *List the number of animals that will experience no more than slight or momentary pain or distress as a result of experimental manipulations or procedures on this protocol.*
- ***Column D:*** *List the number of animals that will potentially experience more than momentary or slight pain or distress that **WILL** be alleviated through the use of anesthetics and/or analgesics.*

- ***Column E:*** *List the number of animals that will experience more than momentary or slight pain or distress that* ***WILL NOT*** *be alleviated or relieved with anesthetics or analgesics.* **If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:**. .

3. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals. Note the DoD-specific database requirements in subparagraph a.

a. Literature Source(s) Searched:

http://www.ntis.gov/products/fedrip.aspxhttp://projectreporter.nih.gov/reporter.cfm

☐ Federal Research in Progress (FEDRIP) http://www.ntis.gov/products/fedrip.aspx

or

☒ Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER) http://projectreporter.nih.gov/reporter.cfm

b. Date of Search:

c. Years Covered By Search:

1987-2021

d. Key Words and/or Search Strategy:

Alternatives to hyperbaric oxygen seizures; alternatives to animal models of hyperbaric oxygen seizures

e. Results of Search:

For "alternatives to hyperbaric oxygen seizures," 6 citations were found using PubMed and 4 citations were found using Web of Science. The projects varied in scope and are not related to this research. For "alternatives to animal models of hyperbaric oxygen seizures," no matches were identified.

****Questions 4-10 refer to the Research Site and Protocol Principal Investigator.****

Information and/or documents required in questions 4-8 should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to BUMED by the Protocol Principal Investigator (PI) or institution staffs. If the Protocol PI prefers that BUMED staff contact the institution to obtain this information and/or documents, the PI must specifically request this action in writing. The Protocol PI should contact BUMED (see contact information on cover page) to arrange for submission of this written request.

4. Institutional Animal Care and Use Committee(s) (IACUC) Approval(s):

Institutional Animal Use Protocol Number:	A224-21-11		
IACUC (initial) approval date:	Nov 19, 2021	Protocol expiration (rewrite) date:	Oct 31, 2024

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

While Duke employs multiple veterinarians, the management of veterinary medicine, animal care, and facility operations is retained by the Director, Division of Laboratory Animal Resources. Duke accepts the American College of Laboratory Animal Medicine's (ACLAM) "Guidelines for Adequate Veterinary Care" (ACLAM 1996) as a core reference document. These guidelines include expectations for

veterinary access to all animals and their medical records, regular veterinary visits to facilities where animals are or may be housed or used, provisions for appropriate and competent clinical, preventive, and emergency veterinary care, and a system for legal animal procurement and transportation. The Division of Laboratory Animal Resources (DLAR) provides husbandry and veterinary care to centralized vivarium operations; husbandry to select satellite facilities, and veterinary care to all satellite and PI-managed facilities. DLAR has a total of six full-time veterinarians, all are 100% FTE. Duke requires that emergency, weekend, and holiday care of animals is performed by qualified personnel. Emergency veterinary care is available after work hours, on weekends, and on holidays. In the event of an emergency, institutional security personnel and fire or police officials are able to reach people responsible for the animals. Notification is enhanced by prominently posting emergency procedures, names, or telephone numbers in animal facilities and by placing them in the security department or telephone center. Emergency procedures for handling special facilities or operations are prominently posted and personnel trained in emergency procedures for these areas. Specifically, DLAR regular animal care staff provide weekend and holiday care in core vivaria, while certified PI staff provide weekend and holiday care in PI-Managed vivaria. Duties covered on weekends and holidays include:

- Mortality-morbidity check
- Food and water check
- Change caging when excessively soiled or wet
- Environment check in all animal rooms
- Medical treatments as prescribed

A Veterinarian and Veterinary Technician (VT) are on call for weekend and holiday coverage. They are responsible for all scheduled treatment procedures during this period along with the VT. The VT performs most scheduled rodent treatments. Key personnel (Veterinarian, Veterinary Technician and Supervisor of Operations Manager) communicate during weekend and holiday periods via pagers and cell phones. A disaster plan that takes into account both personnel and animals has been prepared as part of the overall safety plan for the animal facility. The colony manager or veterinarian responsible for the animals is a member of the appropriate safety committee at Duke, an “official responder” in the institution, and a participant in the response to a disaster. Duke’s disaster plan (DP) covers acts of nature, terrorism, and other catastrophic events. The DP has been developed within Duke and approved by the IO and senior management. The plan takes into account both personnel and animals as part of the overall safety plan for the animal facility. The Director, DLAR is a member of the safety committee, an “official responder” at Duke, and a participant in the response to a disaster. As part of this plan, the Duke police have been involved in the planning and review of the disaster plan.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

☐ Yes ☒ No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

☐ Yes ☒ No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

☐ Yes ☒ No Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific resear

Heath G. Gasier (b) (6)(b) (6) March 18, 2022

(Protocol Principal Investigator Printed Name) (Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

Duke University
Institutional Animal Care and Use Committee
Approval Letter

Office of Animal Welfare Assurance
Campus Mail: (b) (6)(b) (6)(b) (6)
US Mail: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

Voice: (b) (6)(b) (6)
Fax: (b) (6)(b) (6)
https://dacup.duke.edu/

November 19, 2021

RE: **Protocol Registry Number** A224-21-11; **Protocol Title** *The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure*

Dear Dr. Heath Gasier,

On November 18, 2021 the Duke Institutional Animal Care and Use Committee (IACUC) reviewed and **approved** the above referenced protocol.

The terms of this approval are as follows:

- **Approval Period:** This protocol is approved through **October 31, 2024** unless otherwise terminated. If this protocol will continue beyond this approval period, the Duke IACUC will require a complete review by Full Committee Review or Designated Member Review and approval prior reaching the expiration date indicated.
- **Continuing Review(s):** Annual IACUC reviews will be performed if this protocol meets specific criteria that requires an annual review by the Duke IACUC or upon request. In addition, the IACUC can re-review any/all components of this protocol at any time. Reference: Protocol Management (New Protocols, Amendments, and Continuing Review).
- **Principal Investigator Responsibilities:** Use of animals must be in accordance with all applicable federal, funding, local, and institutional regulatory and oversight requirements. This includes but is not limited to the Animal Welfare Act/Regulations, Public Health Services Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, the NIH Grants Policy Statement, VHA Handbook, the DOD Animal Care and Use Review Office requirements, AAALAC International, and Duke Animal Use Policies and SOPs. Please note, certain granting agencies (e.g. Department of Defense) also require their review and approval of the original protocol and any amendments prior to performing the animal work.
- **Approved Number of Animals:** You are responsible for using the minimum number of animals required to obtain valid results. You are required to track and document animal use by utilizing the processes established by the Duke Animal Care and Use Program. Reference: Animal Number Justification and Tracking and associated SOP(s).
- **Protocol Personnel:** All personnel working with animals under this protocol must be appropriately qualified and experienced to perform their role in the animal activity. Reference: Training and Continuing Education for Animal Researchers and Care Staff.
- **Exemptions or departures:** IACUC approved exceptions, exemptions, and/or departures to animal welfare standards must only be performed as described in the approved IACUC protocol.
- **Modifications/Amendments:** Animal activities can only be performed as described in the approved IACUC protocol. New animal activities or other protocol modifications cannot be initiated until final IACUC approval is obtained (e.g., amendment).
- **Reporting requirement:** All unforeseen, adverse, non-compliant, and animal welfare concerns/events should be reported as outlined in the policy IACUC Notifications.
- **Post-approval monitoring:** Post-approval monitoring is required for all IACUC approved animal activities at Duke. More information on post-approval monitoring can be found in the policy Post-approval Monitoring.

Visit the animal program website for links to all the resources indicated, updates, and additional information (https://dacup.duke.edu/). For questions please contact the Duke Office of Animal Welfare Assurance (b) (6)(b) (6); (b) (6)(b) (6)(b) (6)

Please do not hesitate to contact us if there is anything that we can do to facilitate your research.

Sincerely,

(b) (6)

(b) (6)(b) (6)

Duke University Institutional Animal Care & Use Committee
Application for Animal Use

Protocol Registry Number: (Assigned by OAWA) A224-21-11

Previous Protocol Number: (Renewals Only): A023-19-01

PART I: CORE INFORMATION (Sections A - G required for all protocols)
SECTION A: ADMINISTRATIVE

Submission Notes:
* The required submission process is from the PI's 'duke.edu' e-mail address
  (no signatures required if submitted via the PI's 'duke.edu' e-mail address; or if a senior lab member submits from their 'duke.edu' e-mail address and adds the PI to the e-mail as a 'cc').
* Submit all documents directly to IACUC@DUKE.EDU
* Hard copies, scanned hard copies, and FAXed copies of program applications are not accepted.

A1. PERSONNEL INFORMATION

Principal Investigator: Heath Gasier Duke Unique ID: (b) (6)

Dept/Div: (b) (6)(b) (6) Office Phone: (b) (6)(b) (6) Cell Phone: (b) (6)(b) (6)

E-mail Address: (b) (6)(b) (6)(b) (6) Campus Box: (b) (6)

Sponsor: (required when non-Duke faculty or Duke non-faculty PI): Duke Unique ID:

Dept/Div: Office Phone: Cell Phone:

E-mail Address: Campus Box:

Protocol Title: (for this proposal) The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

Grant Title: (if different from protocol) Oxidative tissue damage after HBO2 exposure using selected FDA-approved anti-epileptic drugs (AEDs)

Grant P.I.: (if different from protocol)

Funding Source: Office of Naval Research

Is grant congruency certification required?
◉ NO ○ YES (then submit grant with protocol)

If funded, please provide the SPS #: 3130826

If funded, please provide the Grant #: N00014-18-1-2702

A2. EMERGENCY CONTACTS

○ None. This is a field observation study. No animal holding, housing, management, or control will occur under this protocol.

◉ This is a study where animals will be held, housed, managed, or controlled.

In case animals are observed in distress, the following persons should be contacted:

Primary person to call In case of animal emergencies: (b) (6) Office Phone: (b) (6)(b) (6)

Home Phone: _____ Cell Phone: (b) (6)(b) (6) Pager: _____

Alternative person to call In case of animal emergencies: Heath Gasier Office Phone: (b) (6)(b) (6)

Home Phone: _____ Cell Phone: (b) (6)(b) (6) Pager: _____

Do you have any special instructions to the veterinary staff concerning animal care practices or prohibitions in emergency situations involving animals under this protocol?

- (●) No. Veterinarians should use professional judgement.
- ( ) Yes. As described in Section S: Special Instructions for Emergency Animal Care.

A3. ROLE DELINEATION

NOTE: A Personnel Qualification Form (Part I: Section G) must be completed for every member of the research group listed on this protocol. The form should illustrate skills or training necessary for the roles specified below (e.g., surgeon, anesthetist, phlebotomist, breeder, provides husbandry, observer, etc.).

	NAME	UNIQUE ID	ANIMAL HANDLING ROLE	ROLE IN PROJECT
Add Name	Heath Gasier	(b) (6)	(●) YES ( ) NO	PI (handling, IP injections, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name	(b) (6)		( ) YES (●) NO	Co-I (pathological examination of tissues)
Add Name			(●) YES ( ) NO	LRA I (animal welfare, handling, IP injections, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name			(●) YES ( ) NO	Pulmonary Fellow (handling, IP injections, seizure monitoring, euthanasia, tissue collection)
Add Name			(●) YES ( ) NO	RT II (handling, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name			(●) YES ( ) NO	LRA I (animal welfare)
	DLAR Veterinary Staff (Personnel list maintained in DLAR)	N/A	YES	Provision of controlled substances as described on this protocol, for anesthesia, analgesia, sedation, euthanasia.

A4. COLLABORATING (INCLUDES SUB-CONTRACTING) INSTITUTIONS

Will any facilities other than Duke University (main campus, lemur center, or marine laboratory) or Duke University Medical Center be used for animal use activities (e.g., housing, experimentation, observation, or procedures)?

- (●) No. All work will be performed in Duke University or Durham VAMC facilities.
- ( ) Yes. Work will be performed at another institution.

A5. RENEWAL PROTOCOLS

Does this application renew an existing protocol?

( ) No. This is a new protocol. (●) Yes. This is a renewal protocol. Please complete items `A' and `B' below:

A. Briefly summarize (<150 words) the findings to date as they relate to the use of animals in the retiring protocol:

Of the antiepileptic drugs tested, tiagabine provided the greatest antiseizure protection in mice exposed to hyperbaric oxygen (HBO2).

In addition to its known function of increasing GABAergic neurotransmission, we discovered that pre-treating mice with tiagabine reduced HBO2 mitochondrial DNA oxidation, cellular injury and inflammation, while increasing mitochondrial degradation activation, mitophagy, and a concomitant increase in mitochondrial biogenesis. These responses were primarily documented in the hippocampus and cerebellum. Furthermore, tiagabine did not affect spatial learning and memory in mice over 7 days, assessed by the Barnes maze test.

B. Specify the numbers of animals (by species) that were used In the retiring study (include animals used during the full life of the retiring protocol - generally the last 3 years):

Mouse: Authorized 360
Used 304 Granite = 248 received, 56 on order

C. Please describe any adverse events that occurred with animals on the retiring protocol (if none, please state 'NONE'):

NONE

A6. IMPORTING & EXPORTING ANIMALS, TISSUES, OR ACTIVITIES

Will you be EXPORTING animals, specimens, samples (tissues, blood, organ, etc.), or research products outside of the USA?

- (●) No
- ( ) Yes. I commit to contact Duke Export Controls at (b) (6) prior to exporting any items. The items I am exporting include:

Will you be IMPORTING animals, specimens, samples (tissues, blood, organ, etc.), or research products outside of the USA?

- (●) No
- ( ) Yes. I commit to contact Duke Export Controls at (b) (6) prior to importing any items. The items I am importing include:

A7. MERIT REVIEW

Has this proposal received a scientific merit review?

- ( ) No
- ( ) Yes, this proposal is NIH funded, so an NIH Study Section (or similar) has provided (or will provide) a scientific merit review.
- (●) Yes, this proposal is federally funded (other than NIH), and that agency will provide a scientific merit review.
- ( ) Yes, this proposal is not federally funded. The funding agency has provided a scientific merit review.
- ( ) Yes, this proposal is departmentally funded, and the department has provided a scientific merit review.

The individual who performed the merit review was:

A8. SUPPLEMENTAL SECTIONS INCLUDED IN THIS APPLICATION

- [ ] Section H: Hazardous Agent Use
- [x] Section I: Transportation
- [ ] Section J: Chemical Restraint
- [ ] Section K: Survival Surgical Procedures
- [ ] Section L: Non-Survival Surgical Procedures
- [x] Section M: Non-surgical Procedures

- ☐ Section N: Field Capture / Field Studies
- ☐ Section O: Animal Use in Human Patient Care Areas
- ☒ Section P: Genetically Engineered Animal Use (No Breeding)
- ☐ Section Q: Breeding colonies (Including Genetically Engineered Animals)
- ☐ Section R: Antibody Production
- ☐ Section S: Special Instructions for Emergency Animal Care
- ☒ Section T: PI Managed Animal Facility (holding longer than 12 hours)
- ☐ Section U: Exemptions to The Guide or Routine Animal Care Procedures
- ☐ Section V: Working at the Durham Veterans Administration Medical Center (Duke MOU and VA Protocol Required)
- ☐ Section W: Working at the Duke Lemur Center
- ☐ Section Y: Aquatics, Amphibians, & Reptiles

SECTION B: ANIMAL USE JUSTIFICATION

B1. PURPOSE

Describe in lay terms the purpose of this animal use study (250 words or less):

It is known that increasing the GABA-to-glutamate ratio delays hyperbaric oxygen (HBO2) seizure latency, and this can be accomplished by administering FDA approved antiepileptic drugs (AEDs). We have determined in mice that a single 30 or 60-minute exposure to HBO2 at 5 ATA causes DNA oxidation, inflammation and selective degradation of mitochondria, i.e., mitophagy, mainly in the cerebellum and hippocampus. This is followed by mitochondrial biogenesis and injury resolution within 72-hours. The GABAergic AED tiagabine attenuates acute brain injury and enhances mitochondrial biogenesis. Because exposure to HBO2 increases sensitivity to central nervous system oxygen toxicity (CNS-OT), which is linked to oxidative lung injury, the main objective of this research is to determine how HBO2 and GABA influence repeated exposure-sensitivity to oxidative tissue injury. A secondary objective is to determine the impact of elevated GABA levels on running performance after HBO2. Our hypothesis is that increasing the GABA-to-glutamate ratio will shorten the time course of oxidative injury repair, decreasing repeated HBO2 exposure sensitivity. The rationale is that it will determine if maintaining GABA levels affords protection against oxidative tissue damage with repeated exposure to HBO2 and offer a strong scientific framework for testing GABAergic AEDs in humans. By using male and female mice, we will also establish whether sex hormones are related to GABA levels and HBO2 seizures.

B2. BENEFIT

Describe the potential scientific benefit of the proposed study with respect to human or animal health, the advancement of knowledge, or the good of society (250 words or less):

HBO2 increases the partial pressure of oxygen (PO2) in the lungs, blood and tissues. The pulmonary and central nervous systems are very sensitive to changes in PO2, and high levels can cause oxidative tissue damage that can be lethal. The USN established single-depth oxygen exposure limits from a body of research conducted in the 1970's. The exposure limits serve to reduce the probability of developing CNS-OT that may culminate in tonic-clonic seizures, thus are restrictive for depth and time. This dive schedule constrains military diving capabilities, and may need to be exceeded during operations placing divers at increased risk. For this reason, research has been directed towards understanding the mechanisms of CNS-OT with the goal of finding targeted prevention strategies. This research may lead to new guidelines for the safe use of oxygen during diving operations. This work will also increase our knowledge of the mechanisms of HBO2 seizures and lung injury in the following ways:

1. Establish if GABA is critical in repeated HBO2 exposure sensitivity.
2. Determine if the time course of injury repair influences increased sensitivity to repeated HBO2 exposures.
3. Determine if female sex hormones impact GABA metabolism and increase HBO2 sensitivity.
4. Ensure that increasing GABA levels does not impair post-HBO2 exercise performance.

B3. LITERATURE SEARCH FOR ALTERNATIVES TO POTENTIALLY PAINFUL PROCEDURES OR DISTRESSFUL CONDITIONS

NOTE: The Duke University Medical Center Library has provided a guide "Animals in Research: Searching Alternatives." The IACUC strongly recommends use of this guide to select relevant databases and to develop search strategies for alternatives to painful procedures/distressful conditions affecting animal welfare during the performance of this study.
The link is: http://guides.library.duke.edu/animal_alternatives
For personal assistance, please contact the Biomedical Research Liaison Librarian at iacuc-librarian@duke.edu.

Does the study include activities, procedures, or states of welfare that have the potential for producing pain or distress? Examples: surgical procedures, spontaneous disease, genotype induced disease, tumor development, chronic conditions adversely affecting welfare?

- ○ No. There is no potential for pain/distress.
- ◉ Yes. There is potential for pain/distress.

What are the potentially painful activities or distressful conditions proposed in this animal use application?
(Examples: surgery, toxins, ionizing radiation, implants, tumor development, adjuvants, sepsis, infectious challenge, chronic noise, heat, cold, stressors) Seizures in conscious mice.

A literature search for alternatives to the potentially painful/distressful conditions (listed above) was performed. The details are:

Date (day, month, year) literature search was performed: Oct 5, 2021

Years Covered By The Search (From - To): 1987-2021

Search Strategy / Keywords used in the search:
PubMed
=alternatives to hyperbaric oxygen seizures (6 citations)
=alternatives to animal models of hyperbaric oxygen seizures (0 citations)
Web of Science
=alternatives to hyperbaric oxygen seizures (4 citations)
=alternatives to animal models of hyperbaric oxygen seizures (0 citations)

Databases Searched (**Minimum of two <2> databases required. Check all databases searched.**):

☐ AGRICOLA Data Base	☐ Altweb
☐ AltBib	☐ SCOPUS
☒ PubMed/MEDLINE (is considered one database)	☐ ToxNet
☒ Web of Science	☐ Biosis
☐ Animal Welfare Information Center (AWIC)	☐ Zoological Record
☐ Other (define):	☐ Other (define):

Did the literature search reveal less painful/distressful alternatives to the potentially painful/distressful conditions that are proposed?

- ◉ No alternatives were found.
- ○ Yes, but they are not suitable alternatives for the following reason(s):

	Potentially Painful/Distressful Conditions In This Proposal (match to keywords used)	Write a BRIEF Explanation why the alternatives found to this potentially painful/distressful conditions were not acceptable alternatives.
Add Procedure	Seizures	One citation reported an in vitro model using rat aortic rings, not applicable to this research. The remainder relied on in vivo HBO2 exposures.

B4. LOCATION OF ANIMAL HEALTH / WELL-BEING RECORDS (not research data records)

Records documenting observation of animal health and well-being:

☐ Will not be kept by the research staff. State reason: _____

☐ Will be kept with the animal (next to or near the housing location).

☒ Will be kept at the following location: Building: (b) (6)(b) (6) Room Number: (b) (6)

B5. ANIMAL JUSTIFICATION (applies to all animals)

The justification for using live vertebrate animals rather than alternative means of achieving the research goal is: (check all that apply.)

☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems because:

Our studies are directed towards understanding how HBO2 causes oxidative tissue injury and requires an intact physiological system. No non-animal alternative meets these requirements.

☐ There is not enough information known about the processes being studied to design nonliving models. Explain:

☐ Other (explain):

B6. SPECIES JUSTIFICATION (address each species individually)

Species: Mus musculus

This species was selected for the study because of the following attributes (select all that apply):

☒ A large database exists allowing comparisons with previous data.

Explain: There is a large body of published data using mice in HBO2 as an experimental model for studying oxygen toxicity. The sophistication of genetic tools and the relative ease of mice breeding and housing allows for more accurate and repeatable experiments to determine the cause of HBO2 oxidative tissue damage.

☐ The anatomy or physiology is uniquely suited to the study proposed.

☒ This is the lowest species on the phylogenetic scale that is suitable for the proposed study.

Explain: This is the lowest mammal on the phylogenetic scale that can be used in our project when conscious animals will be used and invasive devices (catheters, electrodes and microdialysis probes) are not needed.

☐ Other attributes. *(details required)*.

Add Species

B7. NUMBER JUSTIFICATION (address each species individually)

Species: Mus musculus

The number of animals requested for this protocol is based on the following (select all that apply):

☒ A statistical estimate of the number required to achieve statistical significance.

Explain: Based on our laboratory's extensive experience, we have found that in HBO2 experiments with conscious mice, there is variability in CNS-OT onset and degree of oxidative tissue damage. In addition, we have traditionally used male mice only, however, this research will examine gender differences because all military occupations are now open to females. Are knowledge of female physiological responses to HBO2 is limited. In order to complete this research, 12 male and 12 female mice are required for each set of experimental paramaters.

☐ The estimated minimum number necessary to achieve the goals of the study in the absence of a statistical estimate.

☐ The number necessary to obtain sufficient tissue or other material for testing or analysis.

☐ The number required to provide sufficient technical training or practice for the number of trainees expected.

☐ The expected or established mortality associated with this procedure.

☐ Other *(details required)*.

Explain:

Add Species

B8. ANIMAL NUMBERS

Adult and/or juvenile (post-weaning) animals only:

NOTE: If this is a renewal protocol and there are animals remaining on the expiring protocol, the number of animals remaining on the expiring protocol (and transferring to the new protocol) must be included in the number of animals requested under the new protocol. Animals on the expiring protocol will be transferred to the new protocol upon activation of the new protocol.

Species: Mus musculus Age or weight range: 2-4 months Number required for 3-year protocol: 592

Distribution of above 3-year number of animals over USDA Categories: See Duke Policy 'Categories of Pain and Distress'

Category B: (Breeding animals)	Category C: (Non-Painful Procedures)	Category D: (Procedures using anesthesia/analgesia)	Category E: (Painful or distressful procedures without anesthesia/analgesia)
	96	64	432

Source of the animals:

NOTE: If transferred from another Duke protocol, provide PI name and protocol number. (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

Add Species

Will embryonic and/or pre-weaning/fledgling animals be used at any time during this protocol? See Duke Policy `Counting Animals`

- (●) No. Only post-weaning animals will be used.
- ( ) Yes. Embryos and/or pre-weaning/fledgling animals will be used.

B9. ANIMAL IDENTIFICATION (Applies to all animals. See Duke's Policy on Animal Identification for more details)

- ( ) Animal identification is not necessary for this protocol.
- (●) Animal Identification is necessary for this protocol.

Specify method(s) below (check all that apply):

- [x] Ear Tagging with a unique numbered tag
- [ ] SQ Radio Tag
- [ ] Collar
- [ ] Tattoo
- [x] Temporary Dye / Ink Marking
- [ ] Ear Punch or notch
- [ ] Toe clipping. (Please review the IACUC policy for toe clipping.)
- [ ] Other Methods:

B10. OVERVIEW OF EXPERIMENTAL DESIGN AND ANIMAL USE TIMELINES

Provide a summary of the overall experimental design. The description should define animal groups, group sizes, anticipated or established mortality for these procedures, and how each group will be tested or used. This section should not include a detailed review of surgery or other activities, but should include the use of any unique drugs or practices:

There will be three independent experiments that employ different experimental designs.
Experiment 1: To determine in C57BL/6 mice if GABA influences repeated HBO2 exposure sensitivity to seizures and lung injury. Male (n = 96) and female (n = 96) C57BL/6J mice (b) (6)(b) (6)) aged 8-12 weeks old will be used in this experiment. There will be three dive profiles. In profile 1, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min. In profile 2, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min and again 48 hours later. In profile 3, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min and again 72 hours later. Forty-eight mice will serve as controls and breathe room air. Mice will be pre-treated (IP injection) 30 min before each HBO2 exposure with TGB (9.4 mg/kg, n = 96) or vehicle (0.9% NaCl, n = 96). Conscious mice are continuously observed during HBO2 for neuromotor convulsions, marking seizure onset.
Category C: 48
Category E: 144

Experiment 2: To determine in mito-QC mice the impact of HBO2 and GABA on recovery from oxidative tissue damage. Male (n = 168) and female (n = 168) mito-QC reporter mice aged 8-12 weeks old will be used in this experiment. Transgenic mito-QC

mice are produced through constitutive knockin of mCherry-GFP-FIS1(101-152) in the mouse Rosa26 locus of C57BL/6 background. These mice will be created by the (b) (6)(b) (6)(b) (6)(b) (6)(b) (6) over 6-7-months. Chimeras will be transferred to the DLAR Breeding Core (A027-21-02 Cleveland) to cross the mice with a Flp-deleter line. Once Frt deletion is confirmed, homozygous mito-QC mice will be bred over 6-12-months and transferred in batches to the (b) (6)(b) (6) (b) (6). The dive profiles and treatment will be the same as in experiment 1, and n = 96 will complete each dive profile. Forty-eight mice will serve as controls and breathe room air. Mice will be pre-treated (IP injection) 30 min before each HBO2 exposure with TGB (9.4 mg/kg, n = 168) or vehicle (0.9% NaCl, n = 168). Conscious mice are continuously observed during HBO2 for neuromotor convulsions, marking seizure onset.
Category C: 48
Category E: 288

Experiment 3: To determine in C57BL/6 mice if GABA impairs running performance after HBO2. Male (n = 32) and female (n = 32) C57BL/6J mice ((b) (6)(b) (6)) aged 8-12 weeks old will be used in experiment 3. Mice will be habituated to a motorized rodent treadmill (Columbus Instruments) before completing a maximal aerobic capacity ($V\dot{O}2max$) test. The treadmill will be located in (b) (6)(b) (6). The treadmill contains a shock grid at the back of the running lane. The shock stimulus will be 0.2 mA, 200 ms pulses, 1 Hz. Habituation will consist of four 10-min running sessions at 0, 3, 6 and 9 meters per min. The treadmill grade will be held at 0 degrees. The $V\dot{O}2max$ protocol will consist of a starting speed of 10 meters per min for 3 min, and then increased by 0.05 meters per sec (3 meters per min). The treadmill grade will be held at 0 degrees. The test will be terminated when the mice are unable to maintain the running speed and are in contact with the electrical grid for 5 consecutive seconds. The modular system contains a fan that provides continuous flow during the running test. At the end of the $V\dot{O}2max$ test, blood lactate will be measured from a small quantity of blood (< 0.010 ml) collected from the tail vein. Mice will be be exposed to air (n = 32) or HBO2 at 3 ATA (n = 32) for 60 min. Mice will be pre-treated (IP injection) 30 min before each air or HBO2 exposure with TGB (9.4 mg/kg, n = 16) or vehicle (0.9% NaCl, n = 16). Mice will complete a second $V\dot{O}2max$ test after the exposure.
Category D: 64

Describe the anticipated sequence of experimental events (timeline) such as breeding, preparation of animals, surgery, testing procedures, collection of tissues, euthanasia, etc.:

All mice will be housed at (b) (6)(b) (6)(b) (6)(b) (6) and (b) (6)(b) (6)(b) (6).

Experiment 1: Mice will be brought to (b) (6) the day before the experiment.
Dive profile 1: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.
Dive profile 2: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be placed back in their cages housed in room (b) (6). Forty-eight hours later, mice will receive an IP injection of TGB or vehicle, and be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.
Dive profile 3: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be placed back in their cages housed in room (b) (6). Seventy-two hours later, mice will receive an IP injection of TGB or vehicle, and be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.

Experiment 2: Once the transgenic mito-QC mice are generated, bred, and transferred to GSRBII, they will be be brought to CRII the day before the experiment. The mice will undergo the same dive profiles described in experiment 1. After the final HBO2 exposure, mice will be placed back in their cages housed in room (b) (6). Mice will be quickly euthanized by inhalation of isoflurane 24- (n = 16, 48- (n = 16) and 72-hours (n = 16) later.

Experiment 3: Mice will be brought to CRII the day before the experiment. Mice will be familiarized to the rodent treadmill and perform a baseline $V\dot{O}2max$ test over one week. On a different day, mice will receive an IP injection of TGB or vehicle and be exposed to air or HBO2 at 3 ATA. After exposure, the mice will complete a second $V\dot{O}2max$ test. Blood will be collected from the tail vein after $V\dot{O}2max$ testing. After the second $V\dot{O}2max$ test and blood collection, mice will be be quickly euthanized by inhalation of isoflurane.

B11. USE OF ANIMALS FOR PERSONNEL TRAINING

Is personnel training the primary purpose of this protocol?

- (●) No, this is not a training protocol. However, I may use small numbers of the approved experimental animals to train my research staff the procedures approved on this protocol.
- ( ) Yes, animals will be used for **non-medical personnel training** (students or others).
- ( ) Yes, animals will be used for **medical personnel training (MD, RN, OD, DC, DVM, etc.)**. Please review Survey Form for Medical Education Using Animals for instructions on completion of the participant survey, and a copy of the survey.
- ( ) Yes, animals will be used for **medical student training**. Please review Survey Form for Medical Education Using Animals for instructions on completion of the participant survey, and a copy of the survey.

For **medical personnel training** and **medical student training,** describe how the use of animals enhances the didactic and simulator components of the training exercise:

B12. GRADES OF CHEMICALS OR SUBSTANCES ADMINISITERED TO ANIMALS:

Note 1: USDA (Policy #3) and The Guide (8th Edition) requires the use of pharmaceutical-grade substances **(medications, diluents, and extenders)** whenever they are available, even in acute procedures. See the Duke Policy on Non-Pharmaceutical Grade Substance Use and the Guidelines for details.

Note 2: Non-pharmaceutical grade chemical compounds may be used in animals only after specific review and approval by the IACUC for reasons such as scientific necessity or non-availability of an acceptable veterinary or human pharmaceutical-grade product. Cost savings is not a justification for using non-pharmaceutical-grade compounds (exceptions for extraordinary costs of substances may be considered).

Note 3: The IACUC may consider the grade, purity, sterility, pH, pyrogenicity, osmolality, stability, site and route of administration, formulation, compatibility, and pharmacokinetics of the chemical or substance to be administered, as well as animal welfare and scientific issues relating to its use when determining whether to approve the use of non-pharmaceutical grade products. Inclusion of this information will assist the Committee's review of your request for use of non-pharmaceutical grade material in animals.

Will this protocol include the use of non-pharmaceutical grade substances, medications, test compounds, vehicles, diluents?

- [x] No. All chemicals and substances used in animals will be pharmaceutical grade.
- [ ] Yes. Some or all chemicals and substances used in animals will be non-pharmaceutical grade. Complete the table below:

	Non-pharmaceutical chemical or substance Required	Scientifically justify why each non-pharmaceutical grade chemical or substance is necessary	Describe the source, purity, formulation, storage, and method of sterilization prior to administering to animals. Supporting documents (e.g., data sheet) should be placed in the document container. (Note: 3)
Add Chem. / Substance			

B13. PHOTOGRAPHY/VIDEOS OF ANIMALS OR TISSUES:

Will images/video be taken of live animals for scientific purposes/publication?

- [ ] No. Proceed to Section B14.
- [x] Yes. I will adhere to the Duke IACUC policy regarding photography of animals. Complete Section B13.

Are animals (tissues) Duke-owned, Client-owned, or Free-ranging?

- [ ] Free-ranging. Note: No approvals are necessary for free-ranging animals.
- [ ] Client-owned. Note: The Client should provide approval to photograph the animals. No Duke approval required.
- [x] Duke-owned. Please complete the following:

Provide the name(s) of the photographer(s) and description of how images/videos will be secured/ protected from unauthorized use:

(b) (6) and (b) (6)(b) (6) may obtain video recordings of the behavior of conscious mice exposed to HBO2. (b) (6) will keep the video recordings under lock and key in her office (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)) and be responsible for their security.

Provide a description and purpose of the photographs/videos, including species (if not all listed in the protocol), and whether the photos are whole body, histologic/fresh/fixed tissues, or radiologic (e.g., CT, PET, MRI):

The video records will allow the investigators to review the behavior of small groups of conscious mice exposed together to hyperbaric oxygen, in order to accurately determine the times onset of CNS toxicity (seizure latency) for individual animals.

B14: UNIQUE STRAINS REGISTRY (MouseBase)

- ☒ I have no unique strains (or substrains) of mice in this proposal.
- ☐ I have registered my unique strains (or substrains) of mice with the Duke MouseBase registry.

SECTION C: HOUSING & PROCEDURE ARRANGEMENTS

C1. DLAR-MANAGED, PI-MANAGED, OR VA-MANAGED HOUSING

NOTE: Confirm housing space availability with DLAR Operations Manager prior to placing an animal order!

Check all that apply:

- ☐ No animal housing will occur at Duke (also includes field studies, collaborating institutions, etc.).
- ☒ DLAR-Managed housing ***NOTE: Contact*** (b) (6)(b) (6) ***to coordinate space requirements prior to ordering animals.***
  - ☐ CCIF-Managed housing ***NOTE: Contact*** (b) (6)(b) (6) ***to coordinate space requirements prior to ordering animals.***
  - ☐ Non-human Primate housing ***NOTE: Contact*** (b) (6) ***to coordinate space requirements prior to ordering animals.***
- ☐ Duke Lemur Center ***NOTE: Contact*** (b) (6)
- ☐ VA-Managed housing (A Duke Protocol is not required, a Duke MOU is required)
  NOTE 1: Contact (b) (6) ***for VA housing arrangements.***
  NOTE 2: Transport between the VA and Duke requires DLAR approval.
  NOTE 3: Holding/housing of, or research with animals at the VAMC requires VA Animal Component of Research Protocol (ACORP).
- ☐ Duke PI-Managed housing ***NOTE: Enter the location on the table below and complete Section T.***

Species: **Mus musculus**

ACTIVITY		BUILDING	ROOM
PI Managed Housing/Holding (or VA-Managed Housing/Holding) (DO NOT complete this row if DLAR Housing)	Greater than 24 hours	(b) (6)(b) (6)(b) (6)(b) (6)	
	Less than 24 hours		
Survival Surgery	Pre-operative preparation		
	Surgery		
	Post-operative care		
Non-Survival Surgery	Pre-operative preparation		
	Surgery		
Nonsurgical Procedures (including euthanasia)		(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)	

ACTIVITY	BUILDING	ROOM
Hazardous Agent Use (location of animals while excreting agent)		
Breeding Colony Activities		

Add Species

C2. SPECIAL HUSBANDRY REQUIREMENTS

Is approval requested for any special husbandry needs? Note that special husbandry needs that are approved must be implemented through direct arrangements with DLAR or Director of the relevant housing facility.

- (●) No. There are no special husbandry requirements.
- ( ) Yes. There are special husbandry needs.

C3. END-OF-STUDY HOUSING

While protocol planning which minimizes animals remaining as single animals for extended periods of time is preferred, on occasion, as experiments approach the end, a single animal may remain. Please answer the following:

- ( ) There will be no end-of-study animals maintained as single animals.
- (●) There may be end-of-study animals which will be maintained as single animals for a limited period of time. I confirm that I will provide an enhanced level of species-specific environmental enrichment for any end-of-study animal being singly housed. I understand that an IACUC Exemption for Single Housing is not required for end-of-study animal(s) housed singly for periods less than 30 days. I shall promptly notify the IACUC if the end-of-study single housing extends beyond 30 days.

SECTION D: SPECIAL CONCERNS FOR ANIMAL USE

D1. EXEMPTION FROM ANIMAL WELFARE STANDARDS

Are there experimental or scientific reasons why any animal on this protocol should be exempted from animal welfare standards?

- (●) No.
- ( ) Yes. An exemption from *Housing Density Standards* is requested in Section U.
- ( ) Yes. An exemption from *Social Housing Standards* is requested in Section U.
- ( ) Yes. An exemption from *Cage Change Frequency Standards* is requested in Section U.
- ( ) Yes. An exemption from *Environmental Conditions (temp, humidity, light, HVAC) Standards* is requested in Section U.
- ( ) Yes. An exemption from *Feed / Water Provision Standards* is requested in Section U.
- ( ) Yes. An exemption from *Radio / Sounds / Noise Standards* is requested in Section U.
- ( ) Yes. An exemption from *Environmental Enrichment Standards* is requested in Section U.
- ( ) Yes. An exemption from *Aquatics Water Quality Standards* is requested in Section U.
- ( ) Yes. An 'OTHER' exemption is requested in Section U.

Explain:

D2. ETHER *(NOTE: The use of ether presents serious animal and researcher safety concerns and requires compelling scientific justification.)*

Will ether be used for euthanasia, anesthesia, or brief sedation?

- (●) No. Ether will not be used.
- ( ) Yes. Ether must be used for scientific reasons.

D3. CONTROLLED SUBSTANCE USE

Will controlled substances be used for anesthesia, restraint, animal management, agent testing, or euthanasia?

- (●) No. Controlled substances will not be used.
- ( ) Yes. Controlled substances will be used, but I will not obtain a controlled substance registration. The DLAR/DLC Technical Services protocol will provide necessary controlled substances for sedation, anesthesia, analgesia, and euthanasia; and DLAR/DLC provision of controlled substances will continue for the duration of this protocol.
- ( ) Yes. Controlled substances will be used. I DO NOT have the registrations at present, but I am/will apply for controlled substance registration. Until my controlled substances registrations are approved by the state & federal agencies, I will use the DLAR/DLC Tech Services protocol for necessary controlled substances used for sedation, anesthesia, analgesia, and euthanasia. An Amendment will be submitted when the state and federal registration numbers are received to transfer authorized controlled substance use off of the DLAR/DLC Tech Services protocol and to my protocol
- ( ) Yes. Controlled substances will be used. A Unit Controlled Substance Registration or an Individual Controlled Substance Registration will provide controlled substances for sedation, anesthesia, analgesia, and euthanasia. DLAR/DLC veterinary staff may provide controlled substance administration at my request. My controlled substance registrations are:

* Registrant Name: (NOTE: The Registrant of the C.S. License / Registration MUST be listed in Protocol Section A-3 (Roles).

* N. Carolina DHHS #: _____ * Federal DEA #: _____

* Location of C.S. storage (bldg/rm):

D4. PHYSICAL RESTRAINT

Will the proposed research require the use of physical restraint (other than short-term hand-held) of awake animals?

- (●) No. Physical restraint of awake animals will not exceed short-term hand restraint.
- ( ) Yes. Physical restraint must be used. I recognize and agree with the following:

- [ ] Restraint devices shall not be considered a normal method of housing.
- [ ] Restraint devices shall not be used as convenience in handling or managing animals.
- [ ] Alternatives to physical restraint have been considered and cannot meet the requirements of my study.
- [ ] The restraint period shall be the minimum required to accomplish my research objectives.
- [ ] Animals shall be acclimated to the devices and personnel prior to actual research use.
- [ ] Animals that fail to adapt shall be removed from the study.
- [ ] Animals in restraint shall be observed at appropriate intervals, as described below.
- [ ] Veterinary care shall be provided if lesions or illnesses associated with restraint are observed.
- [ ] Clear explanation of the purpose of the restraint and its duration shall be provided to personnel involved.

Please address D4a - f below. If the species is a non-human primate, also complete D4g.

D4a. Briefly describe or identify the restraint device:

D4b. Briefly describe the procedure for restraining the animal:

D4c. State the duration of the restraint period:

D4d. Describe the plan for observation of the animal during the period of restraint:

D4e. Describe the plan for animal care and support during the period of restraint to ensure comfort and well-being:

D4f. Describe the procedure for conditioning the animal to the restrain device and procedure so as to minimize potential animal distress during restraint:

D4g. (Non-human primates only) Describe the plan for providing special attention to environmental enrichment during the period of restraint or restricted activity:

D5. WITHHOLDING OF ANESTHETICS OR ANALGESICS

Does this protocol involve procedures that are expected to cause pain, but for which pain-relieving anesthetics and/or analgesics will not be provided?

- ○ No. There are no painful procedures (i.e., no greater pain than would be expected from simple injections).
- ○ No. Anesthetics and/or analgesics will be provided for pain relief.
- ◉ Yes. This protocol includes painful procedures for which anesthetics and/or analgesics must be withheld. Please complete D5a and D5b below.

D5a. Identify the treatment groups and specify the number of animals in each group that will not be provided with pain relief.

Conscious mice breathing 5 ATA oxygen for 60 min (n = 432).

D5b. Detail the scientific reason that requires the withholding of anesthetics and/or analgesics for pain relief.

The purpose of this research is to examine how HBO2 and GABA influences seizures and oxidative tissue damage. The use of anesthetics or analgesics will delay seizure onset or modify neurotoxic behavior. In addition, our goal is to find prevention strategies that can be transitioned into human testing, requiring the use of conscious mice.

D6. ANIMAL WELL-BEING, HUMANE & EXPERIMENTAL ENDPOINTS

According to The Guide, researchers should consider the impact of their procedures upon the animals' well-being. Do you anticipate any animal health complications (e.g. local or systemic infection, physical or physiological impairment, heavy tumor burden, tumor necrosis, malnutrition, dehydration, etc.) arising from the experimental procedures or animal manipulations that are proposed in this protocol?

- ○ No. Animal health complications are not expected. Answer only D6d & D6e.
- ◉ Yes. Animal health complications may occur. Answer D6a through D6e.

D6a. Describe the health complications that are anticipated:

Seizures: Typically last between seconds to a few minutes. We have seen different stages from Stage 1, immobility; Stage 2, forelimb and/or tail extension, rigid posture; Stage 3, repetitive movements, head bobbing. If mice showed extended time of seizure animals will be removed and immediately euthanized. TGB is an FDA approved drug and safe for humans and mice with no reported toxicity.

D6b. Describe the plan for detecting the development of complications and their routine management:

Animals will be constantly monitored during HBO2 for signs of seizure.

D6c. If animals experience complications that are not resolved by the above management plan, specify the action(s) that will be taken (select all that apply).

- ☒ Euthanize the animal.
- ☐ Seek veterinary care from a Duke veterinarian.
- ☐ Withdraw the animal from the study for treatment and recovery according to the following plan (describe):

D6d. According to The Guide, the Principal Investigator, with precise knowledge of both the objectives of the study and the proposed model, should identify, explain and include in the protocol a study endpoint that is both humane and scientifically sound. The experimental endpoint of a study occurs when the scientific aims and objectives have been reached. The humane endpoint is the point at which pain or distress is prevented, terminated or relieved in an experimental animal. The use of humane endpoints contributes to refinement by providing an alternative to experimental endpoints that result in more severe animal pain and distress, including death. Please select the correct response.

- ☒ I anticipate that the experimental endpoints will be reached prior to the humane endpoints
- ☐ I anticipate that humane endpoints will be reached prior to the experimental endpoints. This is necessary because:

D6e. Other than euthanasia, is there the potential for adverse outcomes affecting the animal's well-being (inflammation, inappetence, etc.)?

☐ No, other than euthanasia, there is no potential for adverse outcomes affecting the animal's well-being.

☒ Yes, other than euthanasia, there is the potential for adverse outcomes affecting the animal's well-being. Explain:

HBO2 seizures.

D7. ACCLIMATION AND HABITUATION OF RESEARCH ANIMALS

According to The Guide, habituating animals to routine husbandry or experimental procedures may assist the animal to better cope with a captive environment by reducing stress associated with novel procedures or people. (See Policy on Acclimation) The type and duration of habituation needed will be determined by the complexity / novelty of the procedure. Please select the appropriate response:

Acclimation upon arrival to Duke:

☒ The Policy on Acclimation will be followed.

☐ I am unable to adhere to the Policy on Acclimation. My justification is:

Habituation for Husbandry or Experimental Procedures

☐ Habituation is not necessary. This protocol does not present the animal with novel procedures or people which would require habituation or training to assist the animal with coping.

☒ Habituation / training are performed as part of my research protocol. The methods I will use include:

For involuntary running exercise, mice will be placed on the treadmill set at a 0% incline. The treadmill speed will be increased from 0 to 3 meters per min for 2-6 min (depending on habituation session), increased to 6 meters per min for 2-5 min (depending on habituation session), and then increased to 9 meters per min for 2-4 min (depending on habituation session). Total habituation sessions will be 10-minutes. The shock grid will be set at 0.2 mA, 200 ms pulses, 1 Hz. If during the habituation sessions the mice touch the shock grid for 5 consecutive seconds, the speed will be reduced until the mice resume running.

SECTION E: EUTHANASIA & DISPOSITION

E1. EUTHANASIA:

Please indicate the role of euthanasia in the proposed activity:

☐ Euthanasia is not planned, but if necessary will be performed to prevent animal distress using one of these options:
1) A Duke veterinarian will perform or order to be performed an AVMA-approved method of euthanasia.
2) For situations (e.g. field studies) where no veterinarian is available, the method selected below will be used.

☐ Euthanasia will not be performed. Federal, international, or local permits governing this work do not allow euthanasia.

☒ Animals will be euthanized as part of the experimental protocol. (Specify method below.)

Please specify the method(s) of euthanasia below. Use a separate line for each species studied under this protocol.

Generally Acceptable Methods Of Euthanasia ***(See drop-down list for acceptable options)*:**

	Species	Method	Anesthetic or Injectable Agent / Dose / Route of Administration or Tricaine Concentration
Add Species	Mus musculus	Inhalant anesthetic gas (not ether)	Isoflurane in a bell jar on 4x4 gauze below a raised platform to separate mice for contact with the isoflurane.

NOTE: ***ALL PRIMARY METHODS OF EUTHANASIA require a secondary method*** to ensure non-recovery.
Please select the secondary method(s) that will be used to ensure euthanasia:

- ☒ Bilateral thoracotomy ☐ Decapitation
- ☒ Tissue / organ collection. Specify tissues / organs: _____
- ☒ Other method to ensure death Specify: cervical dislocation

Conditionally Acceptable Methods Of Euthanasia ***(See drop-down list for acceptable options)*:**

	Species	Method	Scientific Justification for Procedure without Anesthesia
Add Species			

Other Methods of Euthanasia ***(consistent with AVMA Panel on Euthanasia recommendations)***

	Species	Method (specify)	Agent / Dose / Route of Administration as Applicabale
Add Species			

Please explain the reason for performing euthanasia by a method other than those identified above as generally or conditionally acceptable:

E2. FINAL DISPOSITION OF ANIMALS

Live animals may be transferred to other approved Duke protocols to facilitate collaborative interactions and reduce overall animal usage and undue wastage. All transfers must be coordinated through DLAR (or DLC for lemurs), unless otherwise specified. In addition to the default method of disposition as described, please also indicate other method(s) of terminating responsibility for the live animals:

- ☐ Live animals may be returned to production / breeding unit.
- ☐ Live animals may be transferred to DLAR for disposition by DLAR as appropriate (requires prior approval by DLAR).
- ☒ Animals will be euthanized by methods specified in section E1 above (Euthanasia).
- ☐ Other (specify): _____

E3. FINAL DISPOSITION OF TISSUES, FLUIDS, OR CARCASSES

Indicate the method(s) of disposing of the carcasses and surplus tissues or fluids (select all that apply):

- ☒ Carcasses of non-biohazardous dead animals will be disposed of by DLAR.
- ☐ Non-radioactive tissues or fluids will be disposed of by the PI.
- ☐ Radioactive carcasses, tissues, or fluids will be disposed of by OESO.
- ☐ Other (specify): _____

E4. SHARING OF TISSUES, FLUIDS, OR CARCASSES

NOTE: This section ONLY APPLIES to animals which are dead prior to collecting the tissues, fluid, or carcasses. Any collection of tissues or fluids from animals which are alive requires specific protocol approval for the collection of tissues or fluids.

Indicate below if you anticipate sharing tissues, fluids, or carcasses post-euthanasia from this protocol:

- ☒ I may share tissues, fluids, or carcasses from my euthanatized animals with Duke researchers.
- ☐ I may share tissues, fluids, or carcasses from my euthanatized animals with non-Duke researchers. Note: Prior to shipping off campus, check with DLAR and OESO for requirements of biological specimen shipment.

SECTION F: PRINCIPAL INVESTIGATOR AGREEMENT

Check each box that is applicable. A checked box indicates agreement by the PI for the statement checked.

- ☒ I will conduct the project in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), and the *Guide for the Care and Use of Laboratory Animals*.
- ☒ I have determined that the research proposed is not unnecessarily duplicative.
- ☒ I confirm that all individuals working on this protocol have been assessed for health risks and are participating in an appropriate Occupational Health & Safety Program. (Note: The Duke Animal Handler Health Review forms are located at http://www.safety.duke.edu; participation in an Occupational Health Program is mandatory).
- ☒ I authorize individuals listed on this application to conduct procedures involving animals and I accept responsibility for their oversight in the conduct of this proposal.
- ☒ I confirm that all individuals listed on this protocol as working with animals have completed the online Animal Handler training or will be required to do so before being permitted to begin work with animals. Further, I certify that those individuals are properly trained, or will receive such training prior to working with animals, in all areas relevant to their assigned work with animals (e.g., biology, handling, and care of the species used; aseptic surgical methods and techniques; the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers; and procedures for reporting animal welfare concerns).
- ☒ For animals under this proposal, I understand that in cases of necessary medical treatment, Duke University veterinarians are authorized to provide any treatment required to sustain life; or if necessary, provide humane euthanasia to prevent unapproved distress and/or pain. I recognize that the veterinary staff will contact me as soon as possible using the emergency contact information that I provide in this application, but I understand that such contact may not always be possible prior to providing treatment or performing euthanasia.
- ☒ I will notify the IACUC (IACUC@duke.edu) of unanticipated outcomes of animal use, including protocol and non-protocol disease or injury. Unanticipated outcomes are generally defined as negatively impacts to animal welfare or well-being. This includes animals at Duke or animals funded by Duke but residing at collaborating institutions.
- ☒ I recognize that veterinary consultation must occur when pain or distress is beyond the level IACUC approved in this protocol, or when my staff are unable to provide interventional control (e.g., euthanasia, immediate removal from the study). For animals held or used on the Duke campus, I will notify a DLAR or DLC veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs.
- ☒ I will obtain approval from the IACUC before initiating any change in the study design or procedures by submitting a request for minor or significant change as appropriate. I understand that work performed without IACUC approval cannot be published with certification of IACUC approval and may result in federally-required reporting of non-compliance.
- ☒ For all USDA Category D (anesthesia / analgesia provided to relieve potential pain) and USDA Category E (pain not relieved by anesthesia / analgesia) animal use procedures, I certify that I have reviewed the pertinent scientific literature and the sources and/or databases noted in this application and found no scientifically acceptable alternative to any of those procedures that would result in less pain or distress.

		Animal Handling Role
PI Name: Heath Gasier	Duke Unique ID (b) (6)	◉ YES ○ NO
Sponsor Name (if applicable):	Duke Unique ID	○ YES ○ NO

Project Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

NOTE: Signature is not necessary when emailed from the PIs 'duke.edu' email address.
If this project has a sponsor, then the sponsor should be listed in the 'cc' box of the email.

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): Gasier, Heath Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)(b) (6)(b) (6)

Dept / Div: (b) (6)(b) (6) Campus Box #: (b) (6) Day Phone #: (b) (6)(b) (6)

Highest Degree Earned: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

Duke Relationship (Check all that apply): (b) (6)

- ○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)
- ◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

- ☒ Animal Handler Health Review clearance through EOHW- Annual requirement
  - ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
  - ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	09/05	☐	☒	09/05	☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☒	09/05	☐	☒	09/05	☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☒	09/05	☐	☒	09/05	☐
Intraperitoneal	☒	09/05	☐	☒	09/05	☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	09/05	☐	☒	09/05	☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☒	09/05	☐	☒	09/05	☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☒	09/05	☐	☒	09/05	☐
Cutdowns	☒	09/05	☐	☒	09/05	☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒	09/15	☐	☒	09/15	☐
Aseptic technique	☒	09/05	☐	☒	09/05	☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	09/15	☐	☒	09/15	☐
Inhalation	☒	09/15	☐	☒	09/15	☐
Injectables	☒	09/05	☐	☒	09/05	☐
Decapitation	☒	09/05	☐	☒	09/05	☐
Cerv. dislocation	☒	09/05	☐	☒	09/05	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	09/05	☐	☒	09/05	☐
Decap.	☒	09/05	☐	☒	09/05	☐
Organ Removal	☒	09/05	☐	☒	09/05	☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

Animal Handling, Texas A&M University, 2005. Investigator Animal Use, Uniformed Services University of the Health Sciences, 2015.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

Sixteen years of experience with mice and rats. HBO2 monitoring, exercise training, non-survival surgeries, blood collection, injections, euthanasia, tissue collection.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6)(b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)(b) (6)(b) (6)

Dept / Div: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6) Campus Box #: (b) (6) Day Phone #: (b) (6)(b) (6)

Highest Degree Earned: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

Duke Relationship (Check all that apply): (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

- (●) I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)
- ( ) I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

- ☐ Animal Handler Health Review clearance through EOHW- Annual requirement
  - ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
  - ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

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- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☐		☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☐		☐	☐		☐
Intraperitoneal	☐		☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☐		☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☐		☐	☐		☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☐		☐	☐		☐
Inhalation	☐		☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☐		☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☐		☐	☐		☐
Decap.	☐		☐	☐		☐
Organ Removal	☐		☐	☐		☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6)(b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)(b) (6)

Dept / Div: (b) (6)(b) (6)(b) (6)(b) (6) Campus Box #: (b) (6) Day Phone #: (b) (6)(b) (6)

Highest Degree Earned: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

Duke Relationship (Check all that apply): (b) (6)

- ○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)
- ◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

- ☒ Animal Handler Health Review clearance through EOHW- Annual requirement
  - ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
  - ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/13	☐	☒	07/01/13	☐
Oral Gavage	☒	07/01/13	☐	☒	07/01/13	☐
Injections						
Intravenous	☒	07/01/13	☐	☒	07/01/13	☐
Intramuscular	☒	07/01/13	☐	☒	07/01/13	☐
Subcutaneous	☒	07/01/13	☐	☒	07/01/13	☐
Intraperitoneal	☒	07/01/13	☐	☒	07/01/13	☐
Intracardiac	☒	07/01/13	☐	☒	07/01/13	☐
Blood collection						
Tail vein	☒	07/01/13	☐	☒	07/01/13	☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☒	07/01/13	☐	☒	07/01/13	☐
Cardiac puncture	☒	07/01/13	☐	☒	07/01/13	☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☒	07/01/13	☐	☒	07/01/13	☐
Neuroleptics	☒	07/01/13	☐	☒	07/01/13	☐
Volatiles	☒	07/01/13	☐	☒	07/01/13	☐
Aseptic technique	☒	07/01/13	☐	☒	07/01/13	☐
Post Surgical Care	☒	07/01/13	☐	☒	07/01/13	☐
Euthanasia						
CO_2	☒	07/01/13	☐	☒	07/01/13	☐
Inhalation	☒	07/01/13	☐	☒	07/01/13	☐
Injectables	☒	07/01/13	☐	☒	07/01/13	☐
Decapitation	☒	07/01/13	☐	☒	07/01/13	☐
Cerv. dislocation	☒	07/01/13	☐	☒	07/01/13	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/13	☐	☒	07/01/13	☐
Decap.	☒	07/01/13	☐	☒	07/01/13	☐
Organ Removal	☒	07/01/13	☐	☒	07/01/13	☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

Training by DLAR staff as required for RACC certification.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have been working with rodents for 20+ years and have experience injecting mice as well as euthanasia.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Treadmill running and HBO2 monitoring. Dr. Heath Gasier will provide the requisite training for these procedures.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6)(b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)(b) (6)(b) (6)(b) (6)

Dept / Div: (b) (6)(b) (6)(b) (6) Campus Box #: (b) (6) Day Phone #: (b) (6)(b) (6)

Highest Degree Earned: (b) (6)(b) (6)(b) (6)(b) (6)

Duke Relationship (Check all that apply): (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

- ○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)
- ◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

- ☒ Animal Handler Health Review clearance through EOHW- Annual requirement
  - ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
  - ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/20	☐	☒	07/20	☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☒	08/18	☐	☒	08/18	☐
Subcutaneous	☒	08/18	☐	☒	08/18	☐
Intraperitoneal	☒	09/20	☐	☒	08/18	☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☐		☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☒	07/20	☐	☒	07/20	☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☒	07/20	☐	☒	07/20	☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒	08/18	☐	☒	08/18	☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	07/20	☐	☒	07/20	☐
Inhalation	☒	07/20	☐	☒	07/20	☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☒	07/20	☐	☒	07/20	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	09/20	☐	☒	07/20	☐
Decap.	☒	09/20	☐	☒	07/20	☐
Organ Removal	☒	09/20	☐	☒	07/20	☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

Indiana University, 2018: rat handling, anesthesia, intubation, ventilation, nonsterile surgical procedures, euthanasia, post-mortem dissection for organ harvesting. Also 2018 online training on rat and mice handling, anesthesia, injections.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

Prior work experience with rats at Indiana University has prepared me for handling rats, rat euthanasia, and post-mortem dissection for organ harvesting. Lab shadowing here at Duke has prepared me for the same procedures in mice.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6)(b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)(b) (6)(b) (6)

Dept / Div: (b) (6)(b) (6) Campus Box #: (b) (6) Day Phone #: (b) (6)(b) (6)

Highest Degree Earned: (b) (6)(b) (6)(b) (6)(b) (6)

Duke Relationship (Check all that apply): (b) (6)

- ○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)
- ◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

- ☒ Animal Handler Health Review clearance through EOHW- Annual requirement
  - ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
  - ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mice					
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/10	☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☒	07/01/10	☐	☐		☐
Intraperitoneal	☒	07/01/10	☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	01/10/21	☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒		☐	☐		☐
Aseptic technique	☒	01/10/21	☐	☐		☐
Post Surgical Care	☒	07/01/10	☐	☐		☐
Euthanasia						
CO_2	☒	07/01/10	☐	☐		☐
Inhalation	☒	07/01/10	☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☐		☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/10	☐	☐		☐
Decap.	☐		☐	☐		☐
Organ Removal	☒	07/01/10	☐	☐		☐

Add skills for additional species

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

I have received my training at the Center for Hyperbaric Medicine and Environmental Physiology over the previous 11 years.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have been working with mice for 10+ years and am skilled in euthanasia, blood and tissue collection.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Treadmill running and HBO2 monitoring. Dr. Heath Gasier will provide the requisite training for these procedures.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): [redacted] Duke Unique ID: [redacted] Date of This Form: Oct 6, 2021

Net ID: [redacted] E-mail Address [redacted]

Dept / Div: [redacted] Campus Box #: [redacted] Day Phone #: [redacted]

Highest Degree Earned: [redacted]

Duke Relationship (Check all that apply): [redacted]

◯ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☐ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse					
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/11	☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☒	12/05/11	☐	☐		☐
Intramuscular	☒	07/01/05	☐	☐		☐
Subcutaneous	☒	12/05/11	☐	☐		☐
Intraperitoneal	☒	12/05/11	☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	12/05/11	☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☒	07/01/05	☐	☐		☐
Cutdowns	☒	07/01/05	☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☐		☐	☐		☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	07/05/16	☐	☐		☐
Inhalation	☒	12/05/11	☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☒	12/05/11	☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/10	☐	☐		☐
Decap.	☒	12/05/11	☐	☐		☐
Organ Removal	☐		☐	☐		☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

I received refresher training on handling and restraint, injections, tail vein blood collection, and CO2 euthanasia (with the secondary methods of cervical dislocation, bilateral thoracotomy, and decapitation for pups) on 12/5/11 with (b) (6) and Durham VAMC vivarium staff. I am also a Duke Certified Research Animal Coordinator.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have over 25 years of experience working with mice at Duke and the Durham VAMC. I have performed all of the procedures in this protocol many times and I am good about keeping up with required refresher training.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION I: TRANSPORTATION

I1. NON-DLAR ANIMAL TRANSPORTATION ON CAMPUS

Will animal be transported between buildings on the Duke campus by other than DLAR vehicles and/or personnel?

- ○ No. DLAR will provide all animal transportation that is required.
- ◉ Yes. Animals will be transported on campus by protocol personnel. Please address the following points:

How many times will any given animal be transported between buildings on campus?

- ◉ One time only.
- ○ More than once.

Describe the type of caging that will be used for transport of animals between locations.

All animals will be transported in DLAR provided caging and/or paperboard container with paperboard lid. Standard mouse box with wire top and filtered cage top.

Indicate the practices and materials that will be used to protect the animals during transit (select all that apply).

- ☒ Cages will be carried by hand.
- ☒ Cages will be moved on a cart or rollable platform.
- ☒ Cages will be moved in a private vehicle (IACUC approval will be required).
- ☒ Cage tops or doors will be secured with a bungee cord or elastic band (no tape)
- ☒ Cages will be covered to prevent visualization of animals during transit.
- ☒ Cages will be moved before or after normal work hours.
- ☐ Other (please describe):

Will the animals be transported by private (non-DLAR) vehicle?

- ○ No.
- ◉ Yes. Please complete the following:

Model of vehicle	(b) (6)(b) (6)
Make (brand) of vehicle	(b) (6)
Tag Number of vehicle	(b) (6)(b) (6)
Location in vehicle where animals will be held (e.g. seat, floor, trunk)	Trunk floor

I2. NON-DLAR ANIMAL TRANSPORTATION OFF CAMPUS

Other than shipment from a commercial vendor, will animals be transported off campus at any time in a non-DLAR vehicle?
Note: Transportation in a private, non-DLAR vehicle requires prior IACUC inspection and approval of the vehicle.

- ◉ No. DLAR will provide all animal transportation that is required.
- ○ Yes. Animals will be transported off campus in a non-DLAR vehicle.

I3. ANIMAL TRANSPORTATION THROUGH PATIENT CARE AREAS

Will animals be transported into or through human patient care areas? (e.g. Duke North, Duke South Clinical Areas, Children's Hospital, etc.)

- ◉ No. Animals will not enter human patient care areas at any time.
- ○ Yes. Section O must be completed. An on-site review of the proposed transport path will be conducted by a Medical Center committee. Contact the Office of Animal Welfare Assurance for additional information.

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)

SECTION M: NON-SURGICAL PROCEDURES

NOTE: Repeat items M1 through M13 *for each species* that will have non-surgical procedures.

The following items M1 - M13 apply to *(identify species)*: Mus musculus

M1. NARRATIVE OF NON-SURGICAL PROCEDURES

Species: Mus musculus

Description of non-surgical procedures:

HYPERBARIC OXYGEN (HBO2) HBOEXPOSURES
After administration of tiagabine, mice will be exposed to elevated atmospheric pressures in a hyperbaric chamber for 60 min. Exposures will be up to 5 ATA. Mice will be placed in individual Plexiglass chambers that receive a continuous supply of 100% oxygen (flow through system). Food and water will not be provided during the exposures. The dive time in the chamber is 60 min of bottom time. There will be travel time to and from the target depth along with a small amount of time on the surface prior to and after the dive when the mice will be in the chambers. The maximum time without food/water will be no longer than 1.5-2.0 hours. The onset of seizures is usually accompanied by unconsciousness, however, because of the possibility that some of these animals may experience distress, they will be designated in USDA pain category E. Mice will be communally housed all other times.

INJECTIONS
Mice will be given a single IP injection of tiagabine or 0.9% NaCl. The injections will be performed with sterile needles (1/2 in 27g) and syringes in the lower abdomen of the mouse.

INVOLUNTARY RUNNING
Mice will be habituated to running on a rodent treadmill and complete a $\dot{V}O_2$max test before HBO2 experiments. Each mouse will be placed on the treadmill the treadmill set at a 0% incline. The treadmill speed will be increased from 0 to 3 meters per min for 2-6 min (depending on habituation session), increased to 6 meters per min for 2-5 min (depending on habituation session), and then increased to 9 meters per min for 2-4 min (depending on habituation session). Total habituation sessions will be 10-minutes. The shock grid will be set at 0.2 mA, 200 ms pulses, 1 Hz. If during the habituation sessions the mice touch the shock grid for 5 consecutive seconds, the speed will be reduced until the mice resume running. On a different day, mice will perform a $\dot{V}O_2$max test. Mice will be placed on the treadmill and the starting speed will be 3 meters per min. Speed will be increased by 0.05 meters per sec (3 meters per min). The treadmill grade will be held at 0 degrees. The test will be terminated when the mice are unable to maintain the running speed and are in contact with the electrical grid for 5 consecutive seconds. On a different day and after HBO2 exposures, mice will repeat the $\dot{V}O_2$max test in identical fashion as the first.

BLOOD LACTATE
After each $\dot{V}O_2$max test, a small volume of blood (< 0.010 ml) will be collect from the tail vein onto lactate test strips inserted into a handheld lactate analyzer. Only 0.003 ml is needed with this analyzer. A repeated measurement may be required if the initial value is outside of the expected lactate concentration.

M2. PRE-PROCEDURE ANIMAL SUPPORT (NOT ANESTHESIA)

Will special pre-procedural care be provided?

○ No. The procedures do not require special pre-procedural care.

◉ Yes. Specify pre-procedural actions that will be taken to prepare animals for the procedure(s) *(select all that apply)*:

- ☐ Physical exam
- ☐ Overnight food withdrawal
- ☐ Body temperature support
- ☐ Clipping of fur
- ☐ CBC (define blood sampling method: _____
- ☐ Chemistry profile (define blood sampling method): _____
- ☐ Ophthalmic ointment to eyes
- ☐ Iodine (or Chlorhexidine) + alcohol skin scrub, 3 alternating cycles
- ☒ Drugs (other than anesthetics and sedatives) or fluids (list agents below):

	Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent	Tiagabine (TGB)	9.4 mg/kg	IP	Once	Single dose, multiple days

M3. PRE-PROCEDURE ANESTHESIA / SEDATION / TRANQUILIZATION

Will pre-procedure anesthesia, sedation or tranquilization be provided to the animals?

- ◉ No. Drugs will not be administered to the animals prior to the procedure(s).
- ○ Yes. Pre-procedure drugs will be used to calm the animals. *(List below)*

	Drug	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Drug					

M4. INTRA-PROCEDURE ANIMAL SUPPORT (NOT ANESTHESIA)

Will special intra-procedure care be provided?

- ◉ No. The procedures do not require special intra-procedural care.
- ○ Yes. Specify intra-procedure care that will be provided to animals during the procedure(s) *(select all that apply)*:

☐ Intravenous fluids

☐ Ophthalmic ointment to eyes

☐ Other (specify): _____

☐ Heat to prevent hypothermia

☐ Cooling to prevent hyperthermia

☐ Other (specify): _____

☐ Drugs (other than anesthetics and sedatives) or fluids (list agents below):

	Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent					

M5. INTRA-PROCEDURE ANESTHESIA OR CHEMICAL RESTRAINT

Will intra-procedure anesthesia or chemical restraint be provided?

- ◉ No. The procedures do not require intra-procedural anesthesia or chemical restraint.
- ○ Yes. The procedure requires chemical restraint as described in Section J or anesthesia as described below:

	Anesthetic Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent					

M6. NEUROMUSCULAR BLOCKING AGENTS (PARALYTICS)

Will neuromuscular blocking agents (paralytics) be used at any time during the procedure?

- ◉ No. Neuromuscular blocking agents will not be used for the procedure.
- ○ Yes. Neuromuscular blocking agents will be used. *(Provide details below)*

	Paralytic Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment	Reversal Agent (if appropriate)
Add Agent						

Please state why the use of paralytic agents during the procedure is necessary.

Please describe how adequate anesthesia will be ensured during the time the animal is undergoing neuromuscular blockade:

M7. MONITORING DEPTH OF ANESTHESIA DURING PROCEDURES

Indicate below the indices that will be used for monitoring animal condition and depth of anesthesia.

- ☐ Respiratory rate / effort
- ☐ Mucous membrane color
- ☐ Body temperature
- ☐ Oxygen saturation
- ☐ Heart rate
- ☐ Blood pressure
- ☐ Capillary refill time
- ☐ EKG
- ☐ Reflex (specify): _____
- ☐ Other (specify): _____
- ☐ Other (specify): _____

Specify the frequency at which the above indices will be recorded: _____

M8. POST-PROCEDURE ANIMAL SUPPORT

Will special post-procedure care be provided?

◉ No. The procedures do not require special post-procedural care.

○ Yes. Specify post-procedure care that will be provided to animals after the procedure(s) *(select all that apply)*:

- ☐ Heat to prevent hypothermia
- ☐ Cooling to prevent hyperthermia
- ☐ Other (specify): _____
- ☐ Intravenous fluids
- ☐ Ophthalmic ointment to eyes
- ☐ Other (specify): _____

M9. MONITORING DURING RECOVERY FROM ANESTHESIA *(if used)*

Indicate below the indices that will be used for post-procedure monitoring of animal condition during recovery from anesthesia (i.e., until sternal recumbancy is regained and maintained):

- ☐ Respiratory rate
- ☐ Mucous membrane color
- ☐ Body temperature
- ☐ Oxygen saturation
- ☐ Heart rate
- ☐ Blood pressure
- ☐ Capillary Refill Time
- ☐ EKG
- ☐ Reflex (specify): _____
- ☐ Other (specify): _____
- ☐ Other (specify): _____

Specify the frequency at which the above indices will be recorded: _____

M10. PAIN MANAGEMENT INTRA- OR POST-PROCEDURE

NOTE: The Duke IACUC encourages the use of pre-emptive analgesia for pain management. Analgesia should be provided as early as possible in the procedure if it is expected to be painful or result in residual pain, ideally before the procedure begins.

Is the procedure expected to cause pain or result in residual pain?

◉ No. The procedure is not expected to cause pain.

○ Yes. Pain during and/or after the procedure is likely.

If pain is expected, will analgesia be provided for pain relief?

○ No. Analgesia will not be provided. (justify the omission of analgesia below)

○ Yes. Analgesia will be provided. (specify details below)

If pain is expected and analgesia will not be provided, please explain why pain relief will be withheld:

If analgesia will be provided for pain relief, please list analgesics and dosing regimens below:

	Analgesic	Timing of Administration	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Analgesic						

M11. SPECIMEN COLLECTION FROM LIVE ANIMALS

Will specimens be collected from living animals during or after the procedure(s)?

○ No. Specimens will not be collected from living animals.

◉ Yes. Define the specimen type and collection details below.

☒ Fluids (e.g., blood, lymph, ascites, CSF, GI fluids, etc.

Fluid type (specify): blood Collection method: tail vein

Volume (mls) per collection: 0.003 Frequency of collection: 2

Method of disposal: Biohazards

☐ Solid Tissues

Tissue type (specify): _____ Collection method: _____

Volume (mm3) per collection: _____ Frequency of collection: _____

Method of disposal: _____

M12. HUMANE ENDPOINTS THAT WILL PROMPT INTERVENTION TO PREVENT CONTINUED PAIN OR DISTRESS

According to The Guide, information that is critical to the IACUC's assessment of appropriate endpoint consideration within a protocol includes precisely defining the humane endpoint (including assessment criteria); the frequency of animal observation; training of personnel responsible for assessment and recognition of the humane endpoint; and the response required upon reaching the humane endpoint. The IACUC has determined that the list below defines the commonly accepted clinical milestones which should be regarded as humane endpoints for most terrestrial animal studies . Choose all of those which are appropriate for the species being used. For each milestone, indicate the action that will be taken. Add other milestones (in the row marked 'other') if applicable for defining the humane endpoints for the proposed study.

Clinical Observation / Milestone	Applicable to my proposal	Frequency of observation	Protocol personnel will perform each of these observations	Response required upon reaching the humane endpoint.	Provide duration (days. weeks. etc.) of monitoring OR a scientific justification for not using the milestones listed
Infection unrelated to the protocol.	No				
Not eating or drinking (will require individual housing to effectively assess)	No				
Decreased fecal and urine output (will require individual housing to effectively assess)	No				
Delayed wound healing (requires checking at least daily until suture removal)	No				
Sudden behavioral change (Ex: aggression, guarding, hiding)	No				
Licking, biting, scratching of the operative / injection site (requires checking at least daily until suture removal)	No				
Poor posture or ambulating difficulty (Ex: tense, tucked-up, stiff gait)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Lost hair coat condition (Ex: ruffled fur, lack of grooming, piloerection)	No				
Sudden activity level change (Ex: restlessness, pacing, reluctance to move)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Unexpected sweating or salivation (Ex: stressed rodents salivate excessively when stressed)	No				
'Painful' facial expression (Ex: grimace, eyes dull, pupils dilated, pinning of ears)	No				
Oculonasal discharge (Ex: rats shed porphyrin pigment when stressed)	No				

Clinical Observation / Milestone	Applicable to my proposal	Frequency of observation	Protocol personnel will perform each of these observations	Response required upon reaching the humane endpoint.	Provide duration (days. weeks. etc.) of monitoring OR a scientific justification for not using the milestones listed
Teeth grinding (Ex: More common sign in rabbits, livestock)	No				
Signs of moderate to severe pain or distress that was not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Body weight loss exceeding 15% of free-feeding body weight relative to an age-matched reference. (Ex: Requires regular <q 48 hours> weighing)					
Self mutilation (requires checking at least daily until suture removal)					
Neurological disorders (e.g., seizures, blindness, ataxia) that were not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Cardiopulmonary disorders (e.g. sudden weakness, vascular collapse, coma) that were not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Abnormal feeding or defecation for 48 hours (e.g., decreased feed or water intake and/or decreased fecal production that is unrelated to the study plan).	No				
Non-weight bearing for 72 hours (e.g., difficulty wa king, inability to maintain upright posture)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Other humane endpoints which will be employed in this project:					
Other humane endpoints which will be employed in this project:					

Add Sections M1 through M13 for another species.

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)

SECTION P: GENETICALLY ENGINEERED (Tg/KO/etc) ANIMAL USE ONLY; NO BREEDING (If genetically engineered animals will be bred at any time during the 3-year life of this protocol, DO NOT complete this section; complete Section Q instead.)

P1. SOURCE OF GENETICALY ENGINEERED ANIMALS

Source of genetically-altered animals:

- ☒ Colony managed by a Duke investigator. Specify source protocol #: A027-21-02
- ☐ Obtained from another institution (or non-Duke investigator). Identify source: _____
- ☐ Purchased from a vendor (e.g. Harlan, Charles River, etc.)

P2. SPECIES AND GENOTYPE

List species to be used, identify genetic lines, and indicate any induction method that is necessary

	Species	Strain / Genotype	Phenotype Induction
Add Species	Mus musculus	mito-QC heterozygotes (express mCherry-GFP with a mitochondrial outermembrane protein, FIS1)	☐ Pharmacologically activated ☐ siRNA ☐ Environmentally activated ☐ Viral induced ☒ Constitutive (gene is always `on') ☐ Other *(specify)*: _____

P3. GENOTYPING

Will genotyping be performed under this protocol?

- ◉ No. Genotyping is not necessary for this protocol.
- ○ Yes. Genotyping will be performed on tissue obtained by the method(s) defined below.

☐ Tail snipping (mice). *Please review the* IACUC policy *for this procedure*.

- ○ The tail snip will be limited to a maximum of 5 mm of the tail tip.
- ○ The tail snip will exceed 5 mm of the tail tip.

  Specify the maximum length of tail tip that will be taken and explain the need for the larger tissue specimen:

- ○ The tail snip will be taken after 21 days of age.
- ○ The tail snip will be taken prior to 21 days of age.

  Justify the delayed genotyping and describe the anesthetic regimen to be used for the procedure:

☐ Oral swabs.

☐ Blood collection.

☐ Other *(describe)*:

P4. PHENOTYPIC EXPRESSION AND CARE PROVISIONS

Briefly describe the genetic alteration of interest:

The use of fluorescent proteins targeting mitochondria allow for selective measurement of mitophagy and architecture. mito-QC (mCherry-GFP-mtFIS1101-153) is a transgenic mouse with a pH sensitive fluorescent mitochondrial indicator that is based on the expression of tandem mCherry-GFP tag fused to an outer mitochondrial protein, mitochondrial fission 1 protein (FIS1). Under normal conditions, the mitochondrial network fluoresces red (mCherry) and green (GFP), but upon mitophagy activation,

GFP fluorescence is quenched by the change in pH and only mCherry remains stable, allowing for the quantification of mitophagy using fluorescence microscopy.

The phenotypic expression of this genetic alteration will likely result in:

- ☒ No morphologic or functional impairment
- ☐ Increased early mortality
- ☐ Physical impairment as follows:
- ☐ Functional deficit as follows:
- ☐ Possible impairments that are presently unknown.

Please describe the monitoring plan that will be used for early detection of potential impairments:

Indicate unique or special animal care needs (e.g., feed, water, temperature, humidly, light, air flow, etc.) that will be required for these genetically-altered animals:

- ☒ No special care is required.
- ☐ Special care needs will be determined by the monitoring plan for detecting potential impairments.
- ☐ Special care must be provided for these animals as follows:

P5. RECORD KEEPING

Indicate the record-keeping system that will be used to document health surveillance and the maintenance of well-being for the genetically-engineered animals.

- ☒ Special care is not required and documentation will be provided by standard DLAR observation records.
- ☐ Special care outlined above will be documented by the record-keeping sheet attached to this application.
- ☐ Special care outlined above will be documented as follows:

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION T: PI MANAGED ANIMAL HOLDING FACILITY

Note: Please refer to the Policy on **PI Managed Housing Facilities** for definitions, approval processes, and other requirements.

T1. REQUEST TO HOLD ANIMALS IN A LABORATORY OR OTHER PI-MANAGED AREA

○ This request is for initial approval of a PI-managed Animal Holding Facility.

◉ This request for renewed approval of an existing PI-managed Animal Holding Facility.

Specify the location of the Animal Holding Facility: Building: (b) (6)

Room(s): (b) (6)

Will any of these animals have ABSL-2 or 3 agents involved? ◉ No ○ Yes

Indicate the type of animal holding or housing for that location:

○ Continual (expectation that one or more animals will be present at any given time).

◉ Frequent (one or more animals often present, but with periods of no animal presence).

○ Sporadic (one or more animals infrequently present,.e.g., 3-4 days/month, with long periods of no animals presence)

Animal species to be held (at each location if multiple rooms are specified):

Mus musculus

Maximum number of cages of animals at this location at any given time: 12 cages

Maximum number of animals at this location at any given time: 48

Maximum duration that any given animal will be held at this location: <4 weeks

Person responsible for the care of animals at this location *(name)*: (b) (6)

According to the NRC 8th Edition of The Guide, testing equipment should be designed in such a way as to allow surface disinfection between studies. Select the correction option:

☒ Yes, testing equipment is designed to allow disinfection between studies

☐ No, testing equipment is not designed to allow disinfection between studies. Please clarify:

T2. JUSTIFICATION FOR ANIMAL HOLDING FACILITY

Please explain the scientific justification for holding animals outside of standard housing facilities and state the reason(s) why standard housing would interfere with achieving the scientific objective(s):

1. Mice will be exposed to HBO2 on more than one occasion separated by 2-3 days, and euthanized up to 1 week after the final HBO exposure.

In addition to the scientific justification provided above, you may include below any other information that you believe important for the IACUC to consider in reviewing this request (optional response).

This study is focused on understanding the effects of repeated HBO2 exposure on the brain and lung, and if the dose of tiagabine used impacts running performance after HBO2. For these reasons, we need to house the animals outside of DLAR.

T3. REQUIRED CLEARANCES

If scientific need is established, the clearances listed below will be required before the IACUC will approve the holding of animals in a PI-managed facility. Either enter the date clearance was obtained or enter "pending" in the date column and e-mail or fax the specific clearance statement to the IACUC when it is obtained.

Clearance	Date Completed	Notes
HVAC assessment (room air flow, pressure)	2/3/2021	**NOTE: These assessments must be done annually**. **For Medical Center buildings**: Contact E&O at (b) (6) **For University buildings**: Contact FMD at (b) (6)
OESO safety audit	2/22/2021	Contact OESO at (b) (6) to have a safety audit.
PI-Managed Husbandry SOP	11/5/2020	Submit Husbandry SOP to (b) (6) at (b) (6)(b) (6)
Complete the appropriate on-line Module: **-PI Managed Housing Training** **NOTE: Modules are the LMS Training site: (https://vmw-lmsweb.duhs.duke.edu/SabaLogin)**	3/16/2018	Notify (b) (6) at (b) (6)(b) (6) of completion of module Note: Module completion required for IACUC Inspection of the proposed housing/holding site.
Adverse Event Plan	8/1/14	Submit Adverse Event Plan to (b) (6) at (b) (6)(b) (6)
IACUC Subcommittee Inspection	10/11/21	Contact (b) (6) at (b) (6) to schedule an IACUC Subcommittee inspection.

**Completed in OAWA Office*

T4. AUTHORIZATIONS PAGE FOR THIS FACILITY'S ADVERSE EVENT PREVENTION, RECOGNITION, & MITIGATION PLAN

Names listed on this section confirms that the individual has reviewed the facility disaster plan and has agreed to implement when determined necessary.

	Facility Contact (Primary contact on line one, secondary contact on line two, etc.)	Duke Unique ID	Emergency Contact Number (generally a cell phone or pager)
Add Name	(b) (6)	(b) (6)	(b) (6)(b) (6)

Duke IACUC

From: Dr Heath Gasier, Ph.D.
Sent: Wednesday, October 27, 2021 1:43 PM
To: Duke IACUC
Subject: RE: Renewal_(b) (4)
Attachments: A-G_Renewal for (b) (4) .pdf; I-Transportation_Renewal for (b) (4) .pdf; M-Non-Surgical_Procedures_Renewal for (b) (4) .pdf; P-Transgenic_or_Knockout_Animal_Use_Renewal for (b) (4) .pdf; T-PI Managed Housing_Renewal for (b) (4) .pdf

(b) (6)

Made the changes, as well as a couple other ones, so please use these documents going forward.

1. (b) (6) will have no handler role (changed).
2. (b) (6) will not be breeding animals (changed).
3. Also changed number of animals used on this protocol (+77 from what is listed; changed).
4. Section T added, will be housed in (b) (6) for <4 weeks.

Heath

From: Duke IACUC (b) (6)(b) (6)(b) (6)
Sent: Wednesday, October 27, 2021 11:41 AM
To: Dr Heath Gasier, Ph.D. (b) (6)
Subject: RE: Renewa (b) (4)

Good morning Dr. Gasier,

Thank you for your IACUC renewal application. I have reviewed it administratively and have the following clarifications:

- Section A3:
  - (b) (6) is indicated as having an animal handler role, however the role described and his Section G indicate a non-animal handler. Please confirm if he will have an animal handling role or not.
  - (b) (6) animal handling role is described as breeding, however there was no section Q attached for animal breeding. If this is needed please submit.
- Section A8 & C1:
  - You've checked the box for the supplemental section T for PI managed housing as well as listed Building (b) (6) as PI managed housing greater than 24 hours.
    - If you will be housing mice in a non-centrally managed location for greater than 24 hours, a Section T is required.
    - Please clarify if you will need PI managed housing or if this location will only be used for non-surgical procedures for less than 24 hours.
    - Reference: PI Managed Housing Facilities policy

Thank you,
(b) (6)
Compliance Liaison, QA & Regulatory Compliance Associate
Office of Animal Welfare Assurance

Duke University Medical Center

(b) (6)

From: Dr Heath Gasier, Ph.D. (b) (6)
Sent: Tuesday, October 26, 2021 3:53 PM
To: Duke IACUC (b) (6)(b) (6)(b) (6)
Subject: Renewal_(b) (4)

This submission is for the upcoming renewal (1/22) for (b) (4), PI Heath G. Gasier, formerly (b) (6)(b) (6)

vr

Heath

Heath G. Gasier, PhD, RD
Assistant Professor in Anesthesiology
Center for Hyperbaric Medicine & Environmental Physiology
Duke University School of Medicine

(b) (6)

(b) (6)

From: (b) (6)
Sent: Wednesday, October 27, 2021 2:12 PM
To: OESO Animal Protocols
Subject: 7N (b) (6)(b) (4)
Attachments: 7N (b) (6)(b) (4) _FCR.pdf

Good afternoon,

Please see the attached new protocol for your review.

Thank you,

(b) (6)
Compliance Liaison, QA & Regulatory Compliance Associate
Office of Animal Welfare Assurance
Duke University Medical Center
(b) (6)

(b) (6)

From: (b) (6)
Sent: Wednesday, October 27, 2021 2:15 PM
To: (b) (6)
Subject: 7N (b) (4) - Vehicle and PI-Managed Housing
Attachments: 7N (b) (4) _FCR.pdf

Good afternoon,

Please see the attached new protocol that has a vehicle and PI Managed housing for facilities clearance.

Thank you,

(b) (6)
Compliance Liaison, QA & Regulatory Compliance Associate
Office of Animal Welfare Assurance
Duke University Medical Center

(b) (6)

(b) (6)

From: (b) (6)
Sent: Wednesday, October 27, 2021 2:50 PM
To: (b) (6)
Cc: Dr Heath Gasier, Ph.D.; (b) (6)
Subject: RE: Facility Review of New Protocol Submission (b) (4)

Thank you, (b) (6)

This resolves my concern. Protocol (b) (4) is now cleared for facilities.

(b) (6)

From: (b) (6)(b) (6)(b) (6)(b) (6)
Sent: Wednesday, October 27, 2021 2:45 PM
To: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Subject: RE: Facility Review of New Protocol Submission: (b) (4)

(b) (6)

That is correct.

Thanks,
(b) (6)
(b) (6)

From: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Sent: Wednesday, October 27, 2021 2:43 PM
To: Dr Heath Gasier, Ph.D. (b) (6)
Cc: (b) (6)(b) (6)(b) (6)(b) (6) Duke IACUC (b) (6)(b) (6)(b) (6)
Subject: Facility Review of New Protocol Submission: (b) (4)

Good afternoon Dr. Gasier,

I am performing a facility review of your recently submitted protocol and have just one clarification:

- Section I includes the (b) (6)(b) (6)(b) (6)(b) (6). In my latest inspection, I noted the tag # changed to (b) (6) Please confirm this tag number is correct.

I have no concerns with your Section T; your PI-managed housing SOP will be updated to reflect the new protocol number.

Thank you,
(b) (6)

(b) (6)
Compliance Liaison
Office of Animal Welfare Assurance
Duke University

(b) (6)

[redacted]

Duke University Institutional Animal Care and Use Committee (DUIACUC)

Occupational and Environmental Safety Office Hazardous Agent Review

PI: Heath G. Gasier Dept/Div: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6) Date Received: 11/09/2021

Project: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

REG#: (b) (4) Housing Location: (b) (6) OAWA Contact: (b) (6)

☒ **Biological Agents** ☒ Cleared For: BSL1 ☐ BSL2 ☐ BSL3 ☐ Transgenic/KO ☐ Micro-Organisms ☐ rDNA/IBC/IRE ☐
☐ Conditional Biological Toxins ☐ Human Material ☐

Comments:
NIH exempt breeding: mCherry-GFP-FIS1(101-152) in the mouse Rosa26 locus of C57BL/6 background.

Reviewed by: (b) (6) Date: 11/09/2021

☒ **Chemical Agents** ☒ Cleared Hazardous Chemicals ☒ Hazardous Drugs ☐
☐ Conditional Fixed Tissue ☐

Comments:

Isoflurane in a bell jar on 4x4 gauze below a raised platform to separate mice for contact with the isoflurane.
[CAS: 26675-46-7] GHS Lookup Tool. STOT-Repeated Exposure, Category 2. Narcotic Effects, Category 3. Hazardous. must be used with an effective mechanism of waste gas scavenging (see The Safe Use of Anesthetic Gases policy found on the Duke Animal Care and Use Program Policies webpage). https://dacup.duke.edu/safe-use-anesthetic-gases (login required). Follow safety guidelines for applicable hazards when using. If using a charcoal canister for scavenging, this must be submitted as chemical waste through OESO, not disposed in the trash. No special animal handling precautions.

Tiagabine (TGB, Gabitril)[145821-59-6]:
Not a hazardous substance or mixture (Sigma SML0035 SDS).

Reviewed by: (b) (6)(b) (6)(b) (6)(b) (6) Date: 11/09/2021

☐ **Radiation Sources** ☐ Cleared Isotopes ☐ X-Ray ☐
☐ Conditional Irradiator ☐ Laser ☐

Comments:

Reviewed by: Date:

☒ **Lab Audit** ☒ Cleared Next Audit Due: 12/17/2021
☐ Conditional

Comments:

Reviewed by: (b) (6) Date: 12/17/2020

OESO Departmental Approval

The referenced protocol has been reviewed and approved for the use of hazardous agents in animals as follows:

- [x] Cleared as written
- [ ] Conditional clearance pending implementation of all recommendations listed above.

Additional Comments

SC 81647: PI change from (b) (6) to (b) (6)(b) (6)

OESO Representative: (b) (6)

Date: 11/09/2021

(b) (6)(b) (6)

From: (b) (6)(b) (6)
Sent: Wednesday, November 10, 2021 2:05 PM
To: (b) (6)(b) (6)
Subject: Review of Protocol (b) (4)(b) (4)
Attachments: (b) (6) Review (b) (4) .docx

(b) (6) ,

Please see the attached veterinary review of your recently submitted animal care and use protocol. You should reply **within the attached document**, **unless otherwise requested**, and send this back to me.
If you could have responses back by November 16, 2021, this will help facilitate review and approval of your protocol at the upcoming IACUC meeting.

Please let me know if there are any questions.

Thank you,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

To: (b) (6)(b) (6)
Registry Number:(b) (4)
Agenda Sequence: 7N
Protocol Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure
Requested Action: Please consider and respond to me as soon as possible

The Duke Institutional Animal Care and Use Committee (IACUC) will review your animal use application shortly. In preparation for a smooth review process and with the hope for a more rapid approval of your proposal, please review and respond to the following veterinary review concerns. **All responses and documents related to this protocol should be sent via the principal investigator's "duke.edu" email account; this will serve as the electronic signature.**

CONCERN NUMBER 1. ROLE DELINEATION (A3)
Please clarify what procedures described in the protocol application that (b) (6) will be responsible for.

CONCERN NUMBER 2. ANIMAL WELL-BEING (D6)
The narrative states that mice that show extended time of seizures will be euthanized, but it is unclear how long this is.
Please clarify what the maximum time of seizure activity that will be allowed prior to euthanasia.

CONCERN NUMBER 3. EUTHANASIA (E1)
Please confirm that all animals that undergo cervical dislocation under anesthesia will also have a visible confirmatory method performed (i.e. bilateral thoracotomy, decapitation).

CONCERN NUMBER 4. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the maximum volume administered for the IP Injections.

CONCERN NUMBER 5. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the method of restraint used for the tail vein blood collection procedures.

CONCERN NUMBER 6. MONITORING AND HUMANE ENDPOINTS (M12)
This section describes daily monitoring for 3 days post HBO2, but the protocol application describes studies where animals will be housed for more than 3 days post HBO2 exposure. Please clarify how animals are monitored for the entire duration that they are housed under the protocol, which is until euthanasia.
How often are animals that undergo multiple HBO2 exposures monitored between exposures?

CONCERN NUMBER 7. MONITORING AND HUMANE ENDPOINTS (M12)
Please confirm that animals are monitored continuously during the time that seizures are anticipated.

While these are the only clarifications requested from the veterinary review, the IACUC Primary Reviewer may contact you for additional clarifications. Please respond as quickly as possible within this document with your clarifications of the concerns identified in this document.

Please **DO NOT** amend your application for animal use, unless specifically requested to do so. Submission of an amended protocol at this stage of the process may impede the overall review, since it will require a complete re-review and may delay approval.

Thank you for your time and effort in this regard. We appreciate your partnering with us to secure a complete review package for the IACUC's consideration. Our goal is a more rapid approval process for your research laboratory, while assuring institutional regulatory compliance, as dictated by the Animal Welfare Act and the Public Health Service (NIH) policy.

Best Wishes,

(b) (6) [redacted]
Division of Laboratory Animal Resources
Duke University Medical Center

(b) (6)(b) (6)

From: (b) (6)(b) (6)
Sent: Tuesday, November 16, 2021 2:32 PM
To: (b) (6)(b) (6) .
Subject: RE: Review of Protocol (b) (4) (Agenda 7N)
Attachments: (b) (6) Review (b) (4) 7N_Resolved.pdf

Hi (b) (6)

Thank you for the responses to the review.

The review concerns are resolved. I have attached the document to reflect this for your protocol records. This will also be placed in and become part of the medical record.

The protocol will be presented at the IACUC meeting this Thursday.

Thanks again,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

From: (b) (6)(b) (6)
Sent: Thursday, November 11, 2021 9:15 AM
To: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Subject: RE: Review of Protocol (b) (4) (Agenda 7N)

Good morning (b) (6)

Attached are the answers to your questions/concerns. Please let me know if you need more detail.

Thank you.

vr

(b) (6)

From: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Sent: Wednesday, November 10, 2021 2:05 PM
To: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Subject: Review of Protoco (b) (6), (b) (4) (Agenda 7N)

(b) (6) ,

Please see the attached veterinary review of your recently submitted animal care and use protocol.

You should reply **within the attached document**, **unless otherwise requested**, and send this back to me.
If you could have responses back by November 16, 2021, this will help facilitate review and approval of your protocol at the upcoming IACUC meeting.

Please let me know if there are any questions.

Thank you,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

To: (b) (6)(b) (6)
Registry Number: (b) (4)
Agenda Sequence: 7N
Protocol Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure
Requested Action: Please consider and respond to me as soon as possible

The Duke Institutional Animal Care and Use Committee (IACUC) will review your animal use application shortly. In preparation for a smooth review process and with the hope for a more rapid approval of your proposal, please review and respond to the following veterinary review concerns. **All responses and documents related to this protocol should be sent via the principal investigator's "duke.edu" email account; this will serve as the electronic signature.**

CONCERN NUMBER 1. ROLE DELINEATION (A3)
Please clarify what procedures described in the protocol application that (b) (6) will be responsible for.
Response: We initially were going to generate the mito-QC mice, but learned that the cost was less to have the Duke Rodent Genetic Engineering Services and DLAR Breeding and Gnotobiotic Core produce and breed them. (b) (6) does the breeding for our center. Because she will not be breeding them, here role will be minimal and consist of monitoring health and well-being, mice identifcation (ear tags), and assist with transportation to (b) (6)

CONCERN RESOLVED

CONCERN NUMBER 2. ANIMAL WELL-BEING (D6)
The narrative states that mice that show extended time of seizures will be euthanized, but it is unclear how long this is.
Please clarify what the maximum time of seizure activity that will be allowed prior to euthanasia.
Response: For 60 min exposures to HBO_2 at 5 ATA, mice are expected to have seizures that generally last several seconds. If a mouse has a tonic clonic seizure for >60 sec, the HBO_2 exposure will be terminated and mouse will be immediately euthanized using isoflurane.

CONCERN RESOLVED

CONCERN NUMBER 3. EUTHANASIA (E1)
Please confirm that all animals that undergo cervical dislocation under anesthesia will also have a visible confirmatory method performed (i.e. bilateral thoracotomy, decapitation).
Response: All mice are euthanized by isoflurane inhalation, followed by cervical dislocation which is a secondary method. This is always confirmed by bilateral thoracotomy since we need to obtain blood and lung tissue.

CONCERN RESOLVED

CONCERN NUMBER 4. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the maximum volume administered for the IP Injections.
Response: 5 µl per gram of body weight.

CONCERN RESOLVED

CONCERN NUMBER 5. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the method of restraint used for the tail vein blood collection procedures.
Response: This will be a two person procedure. One person will restrain the mouse by placing their thumb and index finger on the dorsal neck and grasp the skin with one hand and extend the lower extremities with the other. The animal will be on a flat surface. The other person will collect the blood from the tail. This procedure generally takes 5-15 seconds.

CONCERN RESOLVED

CONCERN NUMBER 6. MONITORING AND HUMANE ENDPOINTS (M12)
This section describes daily monitoring for 3 days post HBO2, but the protocol application describes studies where animals will be housed for more than 3 days post HBO2 exposure. Please clarify how animals are monitored for the entire duration that they are housed under the protocol, which is until euthanasia.
How often are animals that undergo multiple HBO2 exposures monitored between exposures?
Response: Animals will be monitored twice daily (morning and late afternoon).

CONCERN RESOLVED

CONCERN NUMBER 7. MONITORING AND HUMANE ENDPOINTS (M12)
Please confirm that animals are monitored continuously during the time that seizures are anticipated.
Response: Mice are monitored from the time they enter the chamber until the time they are removed, encompassing the entire compression, HBO_2 exposure, and decompression periods.

CONCERN RESOLVED

While these are the only clarifications requested from the veterinary review, the IACUC Primary Reviewer may contact you for additional clarifications. Please respond as quickly as possible within this document with your clarifications of the concerns identified in this document.

Please **DO NOT** amend your application for animal use, unless specifically requested to do so. Submission of an amended protocol at this stage of the process may impede the overall review, since it will require a complete re-review and may delay approval.

Thank you for your time and effort in this regard. We appreciate your partnering with us to secure a complete review package for the IACUC's consideration. Our goal is a more rapid approval process for your research laboratory, while assuring institutional regulatory compliance, as dictated by the Animal Welfare Act and the Public Health Service (NIH) policy.

Best Wishes,

(b) (6) [redacted]
Division of Laboratory Animal Resources
Duke University Medical Center

Duke IACUC

From: Duke IACUC
Sent: Friday, November 19, 2021 9:30 AM
To: (b) (6)(b) (6) .
Cc: (b) (6)
Subject: Approved - IACUC Meeting Results for Protocol (b) (4)
Attachments: 7N (b) (6)(b) (4) _Approved.pdf

Please see your attached approval letter for protocol (b) (4) .

For information on the responsibilities of a PI, please visit the program policy HERE

United States Department of Agriculture
Animal and Plant Health Inspection Service

(b) (6)
(b) (4)(b) (4)(b) (4) Insp_id

Inspection Report

DUKE UNIVERSITY
Office Of Animal Welfare Assurance
(b) (6)(b) (6)(b) (6)
(b) (6)(b) (6)

Customer ID: **863**
Certificate: **55-R-0003**
Site: 001
DUKE UNIVERSITY

Type: ROUTINE INSPECTION
Date: 26-JUL-2021

No non-compliant items identified during this inspection.

This inspection and exit interview were conducted with facility representatives.

Additional Inspectors:

(b) (6)(b) (6), VETERINARY MEDICAL OFFICER

Prepared By: (b) (6), (b)(3)[Title 10 USC 130B]
USDA, APHIS, Animal Care

Date: 27-JUL-2021

Title: VETERINARY MEDICAL OFFICER

Received by Title: Registrant

Date: 27-JUL-2021

United States Department of Agriculture
Animal and Plant Health Inspection Service

Customer: (b) (4) (b) (4)
Inspection Date: 26-Jul-2021

Species Inspected

Cust No	Cert No	Site	Site Name	Inspection
(b) (4)	(b) (4)	001	DUKE UNIVERSITY	26-JUL-2021

(b) (4)

ANIMAL USE OFFICIAL CHECKLIST FOR EXTRAMURAL RESEARCH

- Please complete the following checklist for all extramural research involving animal use and submit it with all applicable documents to the ONR Animal Use Coordinator to forward to the DoD Veterinarian for review/approval. The ONR Animal Use Coordinator will forward the DoD Veterinarians approval letter to the Principal Investigator and Program Manager for inclusion in the PR package to be sent to the Grant Specialist or Contracting Officer.
- If the contract award includes multiple protocols, an Animal Use checklist should be completed for each protocol.

Program Manager:	(b) (6)(b) (6) [redacted]
☒ New Award	☐ Existing Award #
Performer Name:	UNIVERSITY OF SOUTH FLORIDA
Proposal Title:	Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels
Principal Investigator:	JAY DEAN

☒ New Protocol ☐ Amendment NRD #_____

☐ Renewal Protocol (every 3 years): original NRD # _____

YES	N/A	
☒	☐	BUMED DoD Vet Letter
☒	☐	BUMED Appendix (Full or Abbreviated whichever is applicable)
☒	☐	IACUC Approval Letter
☒	☐	IACUC Approved Protocol
☒	☐	USDA Inspection Report (If applicable)
☐	☒	Subcontractors are engaged in the animal use research. *If yes, please list:*
☐	☒	Permit (Field studies)
☐	☒	Using Navy Marine Mammals

Program Officer Review

To the best of my knowledge the information included in this checklist accurately describes the research effort that I am sponsoring.

(b) (6) [redacted]
Program Manager Signature

2/18/20
Date

(b) (6) [redacted]
Animal Use Coordinator Signature

2/19/20
Date

DEPARTMENT OF THE NAVY
BUREAU OF MEDICINE AND SURGERY
7700 ARLINGTON BOULEVARD
FALLS CHURCH VIRGINIA 22042

IN REPLY REFER TO
3900
Ser 22UM24RPV0018
18 April 2022

From: Chief, Bureau of Medicine and Surgery
To: Dr. Thom, Stephen University of Maryland School of Medicine
Via: Chief, Office of Naval Research (Code–34)

Subj: APPROVAL OF PROTOCOL NRD – 1287 Neuroinflammation and glymphatic dysfunction in decompression sickness

Ref: (a) DoD Instruction 3216.01 "Use of Animals in DOD Programs" 20 Mar 19
(b) SECNAVINST 3900.38C "The Care and Use of Laboratory Animals in DOD Programs" 16 Feb 05
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1 – 3)

1. In accordance with references (a) through (c), protocol (0222008) University of Maryland School of Medicine entitled "Neuroinflammation and glymphatic dysfunction in decompression sickness" is granted approval by the Director, Veterinary Affairs and will remain so until significant modification, cancellation, or expiration.

2. Navy animal use research database number NRD - 1287 has been assigned to this protocol. Please reference this number on all future correspondence.

3. Documentation of the following actions must be forwarded to the Director, Veterinary Affairs for review and approval as indicated below:

a. Modifications approved by your IACUC must be reviewed and approved prior to implementation.
b. Verification of continuing reviews conducted by your IACUC.
c. Copies of USDA annual inspection reports.
d. Any regulatory non-compliance evaluation of the animal facility or program by USDA that results in an investigation must be reported within 5 business days.
e. Any change in status with AAALAC, International (gain, loss, or change in category of accreditation); accreditation loss must be reported within 5 business days.
f. Reports to OLAW involving any serious or continuing noncompliance with the PHS Policy, any serious deviation from the provisions of the "Guide for the Care and Use of Laboratory Animals", or any suspension or noncompliance of the activity by the IACUC.
g. Any adverse events, particularly with respect to animal welfare or regulatory compliance or any noncompliance that might impact DoD continued support of the activity.
h. Once the project is completed or if suspended or prematurely terminated for any reason.

4. Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

a. Species used (must be approved by this office)
b. Number of each species used
c. USDA Pain Category for all animals used

5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

6. For further assistance, please contact (b) (6) at (b) (6) or via email (b) (6). You may also contact (b) (6) at (b) (6) or via email at (b) (6).

(b) (6)
Digitally signed by (b) (6)
Date: 2022.04.18 10:55:26 -04'00'

(b) (6)
By direction

Copy:
ONR: (b) (6)
University of Maryland School of Medicine: (Dr. Stephen Thom)

DEPARTMENT OF THE NAVY
BUREAU OF MEDICINE AND SURGERY
7700 ARLINGTON BOULEVARD
FALLS CHURCH VIRGINIA 22042

IN REPLY REFER TO
3900
Ser 20UM24RPV0008
13 February 2020

From: Chief, Bureau of Medicine and Surgery
To: (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Via: Chief, Office of Naval Research (Code–34)

Subj: APPROVAL OF PROTOCOL NRD - 1208

Ref: (a) DoD Instruction 3216.01 "Use of Animals in DOD Programs" 20 September 2019
(b) SECNAVINST 3900.38C "The Care and Use of Laboratory Animals in DOD Programs" 18 June 2019
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1 – 3)

1. In accordance with references (a) through (c), protocols (b) (4) from the University of South Florida entitled "Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channel" is granted approval by the Director, Veterinary Affairs and will remain so until significant modification, cancellation, or expiration.

2. Navy animal use research database number NRD - 1208 has been assigned to this protocol. Please reference this number on all future correspondence.

3. Documentation of the following actions must be forwarded to the Director, Veterinary Affairs for review and approval as indicated below:

a. Modifications approved by your IACUC must be reviewed and approved prior to implementation.
b. Verification of continuing reviews conducted by your IACUC.
c. Copies of USDA annual inspection reports.
d. Any regulatory non-compliance evaluation of the animal facility or program by USDA that results in an investigation must be reported within 5 business days.
e. Any change in status with AAALAC, International (gain, loss, or change in category of accreditation); accreditation loss must be reported within 5 business days.
f. Reports to OLAW involving any serious or continuing noncompliance with the PHS Policy, any serious deviation from the provisions of the "Guide for the Care and Use of Laboratory Animals", or any suspension of the activity by the IACUC.
g. Any adverse events, particularly with respect to animal welfare or regulatory compliance that might impact DoD continued support of the activity.
h. Once the project is completed or if suspended or prematurely terminated for any reason.

4. Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

a. Species used (must be approved by this office)
b. Number of each species used
c. USDA Pain Category for all animals used

5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

6. For further assistance, please contact (b) (6) at (b) (6) or via email: (b) (6) l . You may also contact (b) (6) at (b) (6) or via email at (b) (6) .

(b) (6)

Digitally signed by (b) (6)
Date: 2020.02.13 14:00:45 -05'00'

(b) (6)
By direction

Copy:
ONR: (b) (6)(b) (6)
UOSF: (b) (6)

DEPARTMENT OF THE NAVY
BUREAU OF MEDICINE AND SURGERY
7700 ARLINGTON BOULEVARD
FALLS CHURCH VIRGINIA 22042

IN REPLY REFER TO
3900
Ser 20UM24RPV0036
24 April 2020

From: Chief, Bureau of Medicine and Surgery
To: [redacted]
Via: Chief, Office of Naval Research (Code–34)

Subj: APPROVAL OF PROTOCOL NRD - 1219

Ref: (a) DoD Instruction 3216.01 "Use of Animals in DOD Programs" 20 March 2019
(b) SECNAVINST 3900.38C "The Care and Use of Laboratory Animals in DOD Programs" 18 June 2019
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1 – 3)

1. In accordance with references (a) through (c), protocols (S19154) from the University of California, San Diego entitled "Nitrogen gas uptake and excretion during compression/decompression to simulate diving" is granted approval by the Director, Veterinary Affairs and will remain so until significant modification, cancellation, or expiration.

2. Navy animal use research database number NRD - 1219 has been assigned to this protocol. Please reference this number on all future correspondence.

3. Documentation of the following actions must be forwarded to the Director, Veterinary Affairs for review and approval as indicated below:

a. Modifications approved by your IACUC must be reviewed and approved prior to implementation.
b. Verification of continuing reviews conducted by your IACUC.
c. Copies of USDA annual inspection reports.
d. Any regulatory non-compliance evaluation of the animal facility or program by USDA that results in an investigation must be reported within 5 business days.
e. Any change in status with AAALAC, International (gain, loss, or change in category of accreditation); accreditation loss must be reported within 5 business days.
f. Reports to OLAW involving any serious or continuing noncompliance with the PHS Policy, any serious deviation from the provisions of the "Guide for the Care and Use of Laboratory Animals", or any suspension of the activity by the IACUC.
g. Any adverse events, particularly with respect to animal welfare or regulatory compliance that might impact DoD continued support of the activity.
h. Once the project is completed or if suspended or prematurely terminated for any reason.

4. Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

a. Species used (must be approved by this office)
b. Number of each species used
c. USDA Pain Category for all animals used

5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

6. For further assistance, please contact (b) (6)(b) (6)(b) (6)(b) (6) at (b) (6) or via email: (b) (6)(b) (6)(b) (6)(b) (6). You may also contact (b) (6)(b) (6) (b) (6)(b) (6) at (b) (6) or via email at (b) (6)(b) (6)(b) (6).

(b) (6)(b) (6)
(b) (6)(b) (6)

Digitally signed by (b) (6)(b) (6)(b) (6)(b) (6)
(b) (6)
(b) (6)
Date: 2020.04.24 11:13:44 -04'00'

(b) (6)
By direction

Copy:
ONR: ((b) (6)(b) (6))
UCSD: ((b) (6)(b) (6))

DEPARTMENT OF THE NAVY
BUREAU OF MEDICINE AND SURGERY
7700 ARLINGTON BOULEVARD
FALLS CHURCH VIRGINIA 22042

IN REPLY REFER TO
3900
Ser 22UM24RPV0033
12 July 2022

From: Chief, Bureau of Medicine and Surgery
To: (b) (6) [redacted]
Via: Chief, Office of Naval Research (Code–34)

Subj: APPROVAL OF PROTOCOL NRD - 1288

Ref: (a) DoD Instruction 3216.01 "Use of Animals in DOD Programs" 20 March 2019
(b) SECNAVINST 3900.38C "The Care and Use of Laboratory Animals in DOD Programs" 16 Feb 05
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1 – 3)

1. In accordance with references (a) through (c), protocols (A224-21-11) from Duke University entitled "The role of GABA in susceptibility to oxygen toxicity with repeated HBO2 exposure" is granted approval by the Director, Veterinary Affairs and will remain so until significant modification, cancellation, or expiration.

2. Navy animal use research database number NRD - 1288 has been assigned to this protocol. Please reference this number on all future correspondence.

3. Documentation of the following actions must be forwarded to the Director, Veterinary Affairs for review and approval as indicated below:

a. Modifications approved by your IACUC must be reviewed and approved prior to implementation.
b. Verification of continuing reviews conducted by your IACUC.
c. Copies of USDA annual inspection reports.
d. Any regulatory non-compliance evaluation of the animal facility or program by USDA that results in an investigation must be reported within 5 business days.
e. Any change in status with AAALAC, International (gain, loss, or change in category of accreditation); accreditation loss must be reported within 5 business days.
f. Reports to OLAW involving any serious or continuing noncompliance with the PHS Policy, any serious deviation from the provisions of the "Guide for the Care and Use of Laboratory Animals", or any suspension of the activity by the IACUC.
g. Any adverse events, particularly with respect to animal welfare or regulatory compliance that might impact DoD continued support of the activity.
h. Once the project is completed or if suspended or prematurely terminated for any reason.

4. Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

a. Species used (must be approved by this office)

b. Number of each species used
c. USDA Pain Category for all animals used

5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

6. For further assistance, please contact (b) (6) at (b) (6) or via email: (b) (6) . You may also contact (b) (6) at (b) (6) or via email at (b) (6) .

(b) (6)

Digitally signed by (b) (6)
(b) (6)
Date: 2022.07.12 10:58:06 -04'00'

(b) (6)
By direction

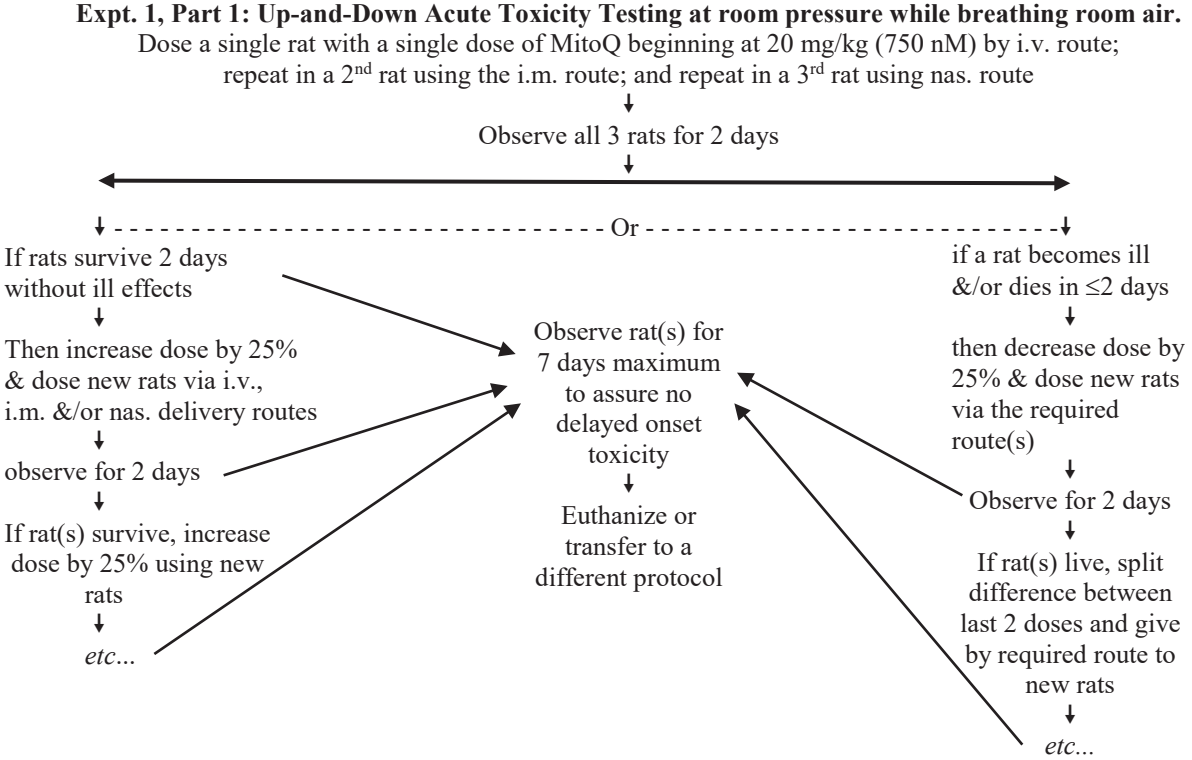
Copy:
ONR: (b) (6)
DU: (b) (6)

Flowcharts for animal experiments in IS0007438, Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels
Major steps in each experiment. Important details are provided in the text of the main document.

Experiment 1 (whole animal, rat) has two sets of experiments.

First, we will determine the appropriate single dose of MitoQ and parenteral route that elevates [MitoQ] in brain tissues within <1 to 2 hr in Sprague-Dawley rats.

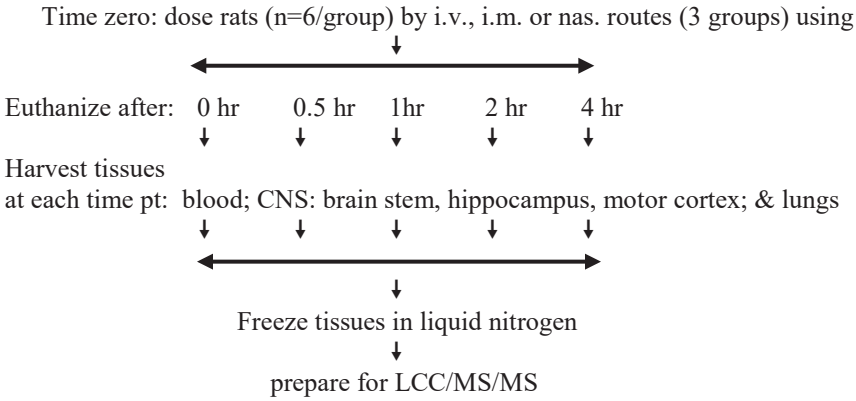
Expt. 1, Part 1: Up-and-Down Acute Toxicity Testing at room pressure while breathing room air.



Up-and-Down Acute Toxicity Testing: based on the literature, we anticipate that 6-10 rats/dosing route will be needed to determine the safe *vs.* toxic dose of MitoQ. Using the highest number, we estimate that 30 rats will be needed to test 3 routes of delivery: [(3 routes: i.v., nas., i.m.) x (1 MitoQ) x (10 rats max. /route)] = 30 rats

---

Expt. 1, Part 2: Liquid Chromatography-Dual Mass Spectroscopy (LC/MS/MS) measurements of MitoQ levels in blood, brain regions & lung tissues as a function time after optimal dosing by i.v., i.m. & nas. routes.



(see above:) At indicated time after dosing, animals are anesthetized using 3-5% isoflurane and an arterial blood sample taken (cardiac puncture under isoflurane anesthesia) to measure the rat's redox status using the redoxSYS analysis meter (40µL blood/sample for analysis). The animal is then euthanized and brain tissues are dissected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Based on a previous study, we plan to use six animals per group: [(6 rats/group) x (3 groups: i.v., nas., i.m.) x (1 substance: MitoQ) x (4 time points: 0.5, 1, 2 & 4 hr post-MitoQ injection)] + (6 rats: baseline group, time 0 (no MitoQ injection)) = 78 SD rats

---

Expt. 2 (whole animal, rat): Expose freely behaving rats to HBO_2 ± MitoQ

Adapt rat to animal chamber inside hyperbaric chamber 1hr/day × 7 days

↓

Dose rat (n=10/group) with either soybean oil vehicle, TPMP, or MitoQ

↓

Place rat in animal chamber inside hyperbaric chamber and ventilate with air for 30 min

↓

Ventilate animal chamber with 1 ATA O_2 (= 100% O_2 at room pressure) for 15 min

↓

Pressurize animal chamber (100% O_2) at ~1 ATG/min to maximum depth of 5 ATA O_2

↓

Continuously observe rat until onset of 1st and 2nd bouts of visible seizures

↓

After 2nd seizures switch to air and begin decompression at ~1 ATG/min back to room pressure

↓

Remove rat from chamber and anesthetize with isoflurane

↓

Take cardiac blood sample under anesthesia

↓

Euthanize by a secondary method
(cervical dislocation, decapitation, or bilateral thoracotomy)

Blood samples will be taken immediately following the dive (cardiac puncture under anesthesia) to measure the animal's redox status using the redoxSYS analysis meter (40µL/sample). Based on our past studies, we plan to use 10 rats/group: [(10 rats/group) x (3 treatments: MitoQ, TPMP & vehicle) = 30 SD rats.

Expt. 3 (whole animal, rat): Using radio telemetric intracerebral microelectrodes, determine locations of "ox tox trigger zones" where seizures originate during exposure to HBO_2 vs. sites of seizure propagation/ amplification, and then determine the effects of mitochondria-targeted antioxidant therapy on regional neural activity using MitoQ.

Give preemptive analgesics & anesthetize rat using isoflurane

↓

Prepare animal for sterile surgery; place rat in supine position, open abdomen, and surgically implant the radio telemetry module with battery; pass telemetry leads through abdominal wall and tunnel them subcutaneously up towards dorsal surface of head; expose telemetry leads near back of skull; open skin over surface of skull

↓

Close abdomen using absorbable sutures for muscle layers and wound clips for skin

↓

Place rat in stereotaxic frame (still anesthetized); exposure surface of skull; determine stereotaxic coordinates for up to 4 microelectrodes and drill openings in skull using a Dremel tool fitted with small burr bit; targeted CNS regions include, for example, brain stem nuclei, hippocampus & motor cortex; reference electrode leads are tied together and positioned over the cerebellum; all electrodes and leads are held in position on skull using dental cement.

↓

Pull skin edges together and close using wound clips

↓

Remove rat from stereotaxic apparatus, place on warming pad and give oxygen and observe until he wakes up; place rat in home cage on top of a heating pad; observe and then return to vivarium once exhibiting normal behaviors; treat animal with post-surgical analgesia

↓

At least 1 week later, remove wound clips and return rat to lab for experiment; i.e., dive in HBO_2 (± MitoQ or TPMP or soybean oil vehicle) until onset of 1st and 2nd bouts of seizures as in Expt. 2 (above)

↓

Return rat to vivarium; at least 1 week later treat rat with 2nd test agent and dive in as described above; each rat dives twice

↓

Euthanize rat after 2nd dive in HBO_2

A pilot study will be done initially to develop the deep intracerebral microelectrode recording capability, which we estimate will require up to 10 animals. The actual study will use 10 rats/group: [(10 rats/group) x (3 groups: MitoQ/TPMP, MitoQ/vehicle, TPMP/vehicle) x 1.15 c.f.] + (10 rats for pilot study) = 45 rats.

END OF WHOLE ANIMAL RAT EXPERIMENTS

BEGIN RAT BRAIN SLICE EXPERIMENTS

Expt. 4 (in vitro brain slice, rat): Determine the effects of MitoQ during exposure to hyperoxia on the rate of production of RONS and $\Delta\Psi_m$ in caudal Solitary Complex (cSC) neurons in brain stem slices

Rats (P1-P21, weanlings & young adults) are euthanized by rapid decapitation

↓

Brain is removed, blocked, and cut into ~400μm thick coronal tissue slices; fluorescence microscopy is done to measure effects of hyperoxia on RONS production and mitochondrial membrane potential, $\Delta\Psi_m$, in cSC neurons.

Assumptions and power analyses used to determine the number of cells needed and animals needed for fluorescence imaging are explained in Section 9. To measure reactive species: [(40 cells/treatment) x (6 rats/40 cells) x (2 dyes) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 40 rats; and to measure mitochondrial membrane potential: [(40 cells/treatment) x (6 rats/40 cells) x (1 dye) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 20 rats

---

Expt. 5 (in vitro brain slice, rat): Determine the effects of MitoQ on O_2-sensitivity or cSC neurons using electrophysiology in rat brain stem slices

Rats (P1-P21, weanlings & young adults) are euthanized by rapid decapitation

↓

Brain is removed, blocked, and cut into ~400μm thick coronal tissue slices; electrophysiology is done to measure effects of hyperoxia ± MitoQ on neuronal electrical signaling.

Estimated numbers of animals required are calculated as follows: [(10 slices/treatment) x (1 rat/cell) x (2 treatments: hyperoxia, MitoQ) x (1.4 c.f. x 2.2 c.f.) x (1 CNS nucleus)] = 62 rats. In Section 9 of the animal protocol, we explain allowances for rat pups born but not used in experiments (13 rats, neonates & weaned rats born but not used in this research (too old for certain experiments)) and the damns required for pups (12 rats, timed-pregnant or nursing dams). In all, 135 rat pups need to be born to yield 40 + 20 + 62 = 122 rat pups for experiments.

END OF RAT BRAIN SLICE EXPERIMENTS & MITOQ STUDY

BEGIN AQUAPORIN-4 (AQP4) MOUSE EXPERIMENTS

Note: *no MitoQ or TPMP will be used in these experiments, only $HBO_2 \pm CO_2$*

AQP4-KO and WT C57/BL6 control mice will be shipped to USF Tampa from Case Western Reserve University, Cleveland, Ohio, Dept. of Physiology and Biophysics. Mice will initially be held in quarantine for 28 days in the COM vivarium before they are used in any experiments. CWRU will have a subcontract with USF on the USF Grant from ONR.

Expt. 6 (whole animal, mice): Effects of HBO_2 on the risk for developing HBO_2-induced seizures in AQP4-KO *vs.* WT mice

Adapt mouse to animal chamber inside hyperbaric chamber 1hr/day × 7 days

↓

Place mouse in animal chamber inside hyperbaric chamber and ventilate with air for 30 min

↓

Ventilate animal chamber with 1 ATA O_2 (= 100% O_2 at room pressure) for 15 min

↓

Pressurize animal chamber (100% O_2) at ~1 ATA/min to maximum depth of 5 ATA O_2

↓

Continuously observe mouse until onset of 1st and 2nd bouts of visible seizures

↓

After 2nd seizures switch to air and begin decompression at ~1 ATA/min back to room pressure

↓

Remove mouse from chamber and return to vivarium

↓

1 week later dive mouse a second time in HBO_2 until onset of 1st and 2nd bouts of visible seizures

↓

Remove mouse from chamber and return to vivarium

↓

1 week later dive mouse a third time in HBO_2 until onset of 1st and 2nd bouts of visible seizures

↓

Remove mouse from chamber and return to vivarium

↓

1 week later dive mouse a fourth and final time in HBO_2 until onset of 1st and 2nd bouts of visible seizures

↓

Remove mouse from chamber and euthanize after 4th dive in HBO_2

THE RATIONALE FOR MULTIPLE DIVES AND SEIZURES is to induce kindling in mice and determine the effect of AQP4 on kindling. The number of mice required for these experiments is: (10 mice/group) x (2 groups: AQP4-KO & WT) = 20 mice total (10 AQP4-KO and 10 WT)

Expt. 7 (whole animal, mice): Effects of $HBO_2 \pm CO_2$ on the risk for developing HBO_2-induced seizures in AQP4-KO *vs.* WT mice

Adapt mouse to animal chamber inside hyperbaric chamber 1hr/day × 7 days

↓

Place mouse in animal chamber inside hyperbaric chamber and ventilate with air for 30 min

↓

Ventilate animal chamber with 1 ATA O_2 (= 100% O_2 at room pressure) for 15 min

↓

Pressurize animal chamber (100% O_2) at ~1 ATA/min to maximum depth of 5 ATA O_2

↓

Continuously observe mouse until onset of 1st and 2nd bouts of visible seizures

↓

After 2nd seizures switch to air and begin decompression at ~1 ATA/min back to room pressure

↓

Remove mouse from chamber and return to vivarium

↓

1 week later dive mouse a second time as before until onset of 1st and 2nd bouts of visible seizures only this time use HBO_2 + CO_2 (randomize order of HBO_2 alone and HBO_2 + CO_2 between animals)

↓

Remove mouse from chamber and return to vivarium

↓

Remove mouse from chamber and euthanize after 2nd dive in HBO_2

Expt. 8 (whole animal, mice): Effects of hyperbaric oxidative preconditioning on AQP4 expression and the risk for developing HBO_2-induced seizures in WT mice.

Two mice at a time are pretreated (preconditioned) with a sublethal level of HBO_2:

2.5 ATA HBO_2 x 90-min x 4 treatments; i.e.

2.5 ATA O_2 x 90-min

↓ (4.5 hr 1 ATA air)

2.5 ATA O_2 x 90-min

↓ (4.5 hr 1 ATA air)

2.5 ATA O_2 x 90-min

↓ (4.5 hr 1 ATA air)

2.5 ATA O_2 x 90-min

↓ (4 hr 1 ATA air)

Remove mice from preconditioning pressure chamber & place 1 mouse in animal chamber inside hyperbaric chamber and ventilate with air for 0.5 hr

} 24 hr in MDL 1026

↓

Ventilate animal chamber with 1 ATA O_2 (= 100% O_2 at room pressure) for 15 min

↓

Pressurize animal chamber (100% O_2) at ~1 ATG/min to maximum depth of 5 ATA O_2

↓

Continuously observe mouse until onset of 1st and 2nd bouts of visible seizures

↓

After 2nd seizures switch to air and begin decompression at ~1 ATG/min back to room pressure

↓

Remove mouse from chamber and return to vivarium

↓

Remove mouse from chamber; euthanize by trans-cardial perfusion & fixation; Harvest brain tissue for AQP4 immunohistochemistry processing

↓

Clean animal chamber and insert 2nd preconditioned mouse

↓

Repeat HBO_2 dive experiment for 2nd preconditioned mouse, euthanize & harvest brain tissue for AQP4 immunostaining

Three groups of WT mice will be studied; 1) mice preconditioned using 2.5 ATA HBO_2 (describe above in flowchart); control WT mice preconditioned with normoxic hyperbaric pressure (14% O_2 + balance Helium = 0.21 ATA O_2 + 0.79 ATA He), and another group of control WT mice maintained in the preconditioning chamber for 24h with ventilation using 1 ATA air. The number of mice needed for this experiment is: (3 groups of preconditioned WT mice: HBO, normoxic hyperbaric pressure, and 1 ATA air) x (10 mice/group) = 30 WT mice

OMB Number: 4040-0001
Expiration Date: 12/31/2022

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE

State Application Identifier

1. TYPE OF SUBMISSION

☐ Pre-application ☒ Application ☐ Changed/Corrected Application

4. a. Federal Identifier N00014

b. Agency Routing Identifier 342 [Chapman, Sandra]

c. Previous Grants.gov Tracking ID

2. DATE SUBMITTED

Applicant Identifier 271024

5. APPLICANT INFORMATION

Organizational DUNS: 0443877930000

Legal Name: Duke University

Department:

Division:

Street1: Office of Research Administration

Street2: (b) (6) [redacted]

City: Durham

County / Parish:

State: NC: North Carolina

Province:

Country: USA: UNITED STATES

ZIP / Postal Code: 27705-4677

Person to be contacted on matters involving this application

Prefix:

First Name: (b) (6) [redacted]

Middle Name:

Last Name: (b) (6) [redacted]

Suffix:

Position/Title: (b) (6) [redacted]

Street1: Office of Research Administration

Street2: (b) (6) [redacted]

City: Durham

County / Parish:

State: NC: North Carolina

Province:

Country: USA: UNITED STATES

ZIP / Postal Code: 27705-4677

Phone Number: (b) (6) [redacted]

Fax Number:

Email: gcmail@mc.duke.edu

6. EMPLOYER IDENTIFICATION *(EIN) or (TIN):* 56-053-2129

7. TYPE OF APPLICANT: O: Private Institution of Higher Education

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. NAME OF FEDERAL AGENCY: Office of Naval Research

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER: 12.300

TITLE: Basic and Applied Scientific Research

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

12. PROPOSED PROJECT:

Start Date: 01/01/2022

Ending Date: 12/31/2024

13. CONGRESSIONAL DISTRICT OF APPLICANT

NC-004

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name: Heath Middle Name:

Last Name: Gasier Suffix:

Position/Title: Assistant Professor of Anesthesiology

Organization Name: Duke University

Department: Anesthesiology Division: GVT

Street1: (b) (6)

Street2: (b) (6)

City: Durham County / Parish:

State: NC: North Carolina Province:

Country: USA: UNITED STATES ZIP / Postal Code: 27710-1000

Phone Number: (b) (6) Fax Number:

Email: (b) (6)

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	860,828.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	860,828.00
d. Estimated Program Income	0.00

16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

☒ **I agree**

**The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL (Disclosure of Lobbying Activities) or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: First Name: (b) (6) Middle Name:

Last Name: (b) (6) Suffix:

Position/Title: (b) (6)

Organization: Duke University

Department: Division:

Street1: Office of Research Administration

Street2: (b) (6)

City: Durham County / Parish:

State: NC: North Carolina Province:

Country: USA: UNITED STATES ZIP / Postal Code: 27705-4677

Phone Number: (b) (6) Fax Number:

Email: gcmail@mc.duke.edu

Signature of Authorized Representative	Date Signed
(b) (6)	09/30/2021

20. Pre-application Add Attachment | Delete Attachment | View Attachment

21. Cover Letter Attachment Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0001
Expiration Date: 12/31/2022

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Heath Middle Name:
* Last Name: Gasier Suffix:
Position/Title: Assistant Professor of Anesthesiology Department: Anesthesiology
Organization Name: Duke University Division: GVT
* Street1: (b) (6)
Street2: (b) (6)
* City: Durham County/ Parish:
* State: NC: North Carolina Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 27710-1000
* Phone Number: (b) (6) Fax Number:
* E-Mail: (b) (6)
Credential, e.g., agency login: (b) (6)
* Project Role: PD/PI Other Project Role Category:
Degree Type:
Degree Year:

*Attach Biographical Sketch	1235-Biosketch_Gasier.pdf	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support	1236-Support_Gasier.pdf	Add Attachment	Delete Attachment	View Attachment

PROFILE - Senior/Key Person 1

Prefix: (b) (6) * First Name: (b) (6) Middle Name:
* Last Name: (b) (6) Suffix:
Position/Title: (b) (6) Department: Anesthesiology
Organization Name: Duke University Division:
* Street1: (b) (6)
Street2: (b) (6)
* City: Durham County/ Parish:
* State: NC: North Carolina Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 27710-1000
* Phone Number: (b) (6) Fax Number:
* E-Mail: (b) (6)
Credential, e.g., agency login: (b) (6)
* Project Role: Co-Investigator Other Project Role Category:
Degree Type:
Degree Year:

Attach Biographical Sketch	1237-Biosketch(b) (6) .pdf	Add Attachment	Delete Attachment	View Attachment
Attach Current & Pending Support	1238-Support_(b) (6).pdf	Add Attachment	Delete Attachment	View Attachment

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 12/31/2022

1. Are Human Subjects Involved? ☐ Yes ☒ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☒ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number: D16-00123

3. Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract 1239-Abstract.pdf Add Attachment Delete Attachment View Attachment

8. Project Narrative 1240-Project Narrative.pdf Add Attachment Delete Attachment View Attachment

9. Bibliography & References Cited 1241-Bibliography & References Cited. Add Attachment Delete Attachment View Attachment

10. Facilities & Other Resources 1242-Facilities & Other Resources.pdf Add Attachment Delete Attachment View Attachment

11. Equipment Add Attachment Delete Attachment View Attachment

12. Other Attachments Add Attachments Delete Attachments View Attachments

OMB Number: 4040-0019
Expiration Date: 02/28/2022

Project Abstract Summary

This Project Abstract Summary form must be submitted or the application will be considered incomplete. Ensure the Project Abstract field succinctly describes the project in plain language that the public can understand and use without the full proposal. Use 4,000 characters or less. Do not include personally identifiable, sensitive or proprietary information. Refer to Agency instructions for any additional Project Abstract field requirements. If the application is funded, your project abstract information (as submitted) will be made available to public websites and/or databases including USAspending.gov.

Funding Opportunity Number

N00014-21-S-B001

CFDA(s)

12.300

Applicant Name

Duke University

Descriptive Title of Applicant's Project

The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

Project Abstract

Hyperbaric oxygen (HBO2) increases the partial pressure of oxygen (PO2) in the lungs, blood and tissues. The pulmonary and central nervous systems are very sensitive to changes in PO2, and high levels can cause oxidative tissue damage that can be lethal. The USN established single-depth oxygen exposure limits that serve to reduce the probability of developing central nervous system oxygen toxicity (CNS-OT) that may culminate in tonic-clonic seizures, thus are restrictive for depth and time. For this reason, research has been directed towards understanding the mechanisms of CNS-OT with the goal of finding targeted prevention strategies. It is known that increasing the GABA-to-glutamate ratio delays seizure latency, and this can be accomplished by administering FDA approved antiepileptic drugs (AEDs). We have determined in mice that a single 60-minute exposure to HBO2 at 5 ATA causes DNA oxidation, inflammation and selective degradation of mitochondria, i.e., mitophagy, mainly in the cerebellum and hippocampus. This is followed by mitochondrial biogenesis and injury resolution within 72-hours. The AED tiagabine (TGB) attenuated acute brain injury and enhanced mitochondrial biogenesis. Because exposure to HBO2 increases sensitivity to CNS-OT, which is linked to oxidative lung injury, the main objective in this application is to determine how HBO2 and GABA influence repeated exposure-sensitivity to oxidative tissue injury. A secondary objective is to determine the impact of elevated GABA levels on running performance after HBO2. Our central hypothesis is that increasing the GABA-to-glutamate ratio will shorten the time course of oxidative injury repair, decreasing repeated HBO2 exposure sensitivity. The rationale is that it will determine if maintaining GABA levels affords protection against oxidative tissue damage with repeated exposure to HBO2 and offer a strong scientific framework for testing GABAergic AEDs in humans. To test our hypothesis and attain our objective, the following three specific aims will be pursued: 1) Determine in C57BL/6 mice if GABA influences repeated HBO2 exposure sensitivity to seizures and lung injury. 2) Determine in mito-QC mice the impact of HBO2 and GABA on recovery from oxidative tissue damage. 3) Determine in C57BL/6 mice if increased GABA impairs running performance after HBO2. To accomplish this research, we will use wild type and transgenic mice with fluorescent labels for mitochondrial architecture and mitophagy (mito-QC) and expose them to single or repeated HBO2 exposures at 5 ATA. Running performance will be tested after HBO2 at 3 ATA. GABA metabolism and oxidative injury in the brain and lung will be measured by ultra-performance liquid chromatography-tandem mass spectrometry, immunohistochemistry and immunofluorescence, immunoblotting and assays. The proposed research is significant because it will lay the groundwork for testing GABAergic AEDs in humans performing work in HBO2. The proximate expected outcomes of this work is knowledge of how GABA influences the time course of oxidative injury repair in the brains and lungs of mice exposed to repeated HBO2. The results will have an important positive impact immediately because they will establish new function(s) of GABA, and potentially lead to increased oxygen exposure limits. The proposed research is relevant to warfighters because breathing 100% oxygen during diving operations can impair work performance and mitigation approaches are needed. This proposal addresses two of Undersea Medicine & Performance Concentration Areas: mitigation of hyperbaric oxygen toxicity and diver performance optimization.

“Approved for Public Release”

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 12/31/2022

ORGANIZATIONAL DUNS: 0443877930000 **Enter name of Organization:** Duke University

Budget Type: ☒ Project ☐ Subaward/Consortium **Budget Period: 1** **Start Date:** 01/01/2022 **End Date:** 12/31/2022

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Dr.	Heath		Gasier		(b) (4)	2.40			(b) (4)	(b) (4)	(b) (4)
Project Role: PD/PI											
(b) (6)	[redacted]		[redacted]		(b) (4)	2.40			(b) (4)	(b) (4)	(b) (4)
Project Role: Co Investigator											

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person (b) (4)

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Technician	0.12			(b) (4)	(b) (4)	(b) (4)
1	Technician	0.24			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	2.40			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	1.80			(b) (4)	(b) (4)	(b) (4)
1	Research Technician II	2.40			(b) (4)	(b) (4)	(b) (4)
5	**Total Number Other Personnel**					**Total Other Personnel**	(b) (4)

Total Salary, Wages and Fringe Benefits (A+B) (b) (4)

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees — **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other Expenses	19,540.00
9.		
10.		
	Total Other Direct Costs	19,540.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	107,904.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	(b) (4)	(b) (4)	(b) (4)
		Total Indirect Costs	(b) (4)

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	173,725.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	173,725.00

L. Budget Justification

(Only attach one file.) 1234-Budget Justification.pdf

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 12/31/2022

ORGANIZATIONAL DUNS: 0443877930000 **Enter name of Organization:** Duke University

Budget Type: ☒ Project ☐ Subaward/Consortium **Budget Period: 2** **Start Date:** 01/01/2023 **End Date:** 12/31/2023

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Dr.	Heath		Gasier		(b) (4)	4.80			(b) (4)	(b) (4)	(b) (4)
Project Role: PD/PI											
(b) (6)	[redacted]		[redacted]		(b) (4)	4.80			(b) (4)	(b) (4)	(b) (4)
Project Role: Co Investigator											

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person (b) (4)

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Technician	0.18			(b) (4)	(b) (4)	(b) (4)
1	Technician	0.36			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	4.80			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	3.60			(b) (4)	(b) (4)	(b) (4)
1	Research Technician II	3.60			(b) (4)	(b) (4)	(b) (4)
5	**Total Number Other Personnel**					**Total Other Personnel**	(b) (4)

Total Salary, Wages and Fringe Benefits (A+B) 175,015.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	3,455.00
2. Foreign Travel Costs	
Total Travel Cost	3,455.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees — **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	35,310.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other Expenses	25,224.00
9.		
10.		
	Total Other Direct Costs	60,534.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	239,004.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	(b) (4)	(b) (4)	(b) (4)
		Total Indirect Costs	(b) (4)

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	384,796.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	384,796.00

L. Budget Justification

(Only attach one file.) 1234-Budget Justification.pdf

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 12/31/2022

ORGANIZATIONAL DUNS: 0443877930000 **Enter name of Organization:** Duke University

Budget Type: ☒ Project ☐ Subaward/Consortium **Budget Period: 3** **Start Date:** 01/01/2024 **End Date:** 12/31/2024

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Dr.	Heath		Gasier		(b) (4)	4.80			(b) (4)	(b) (4)	(b) (4)
Project Role: PD/PI											
(b) (6)	[redacted]		[redacted]		(b) (4)	4.80			(b) (4)	(b) (4)	(b) (4)
Project Role: Co Investigator											

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person (b) (4)

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Technician	0.06			(b) (4)	(b) (4)	(b) (4)
1	Technician	0.12			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	6.00			(b) (4)	(b) (4)	(b) (4)
1	Lab Research Analyst I	1.80			(b) (4)	(b) (4)	(b) (4)
1	Research Technician II	3.60			(b) (4)	(b) (4)	(b) (4)
5	**Total Number Other Personnel**					**Total Other Personnel**	(b) (4)

Total Salary, Wages and Fringe Benefits (A+B) 173,931.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	3,632.00
2. Foreign Travel Costs	
Total Travel Cost	3,632.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees — **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	9,005.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Other Expenses	1,200.00
9.		
10.		
	Total Other Direct Costs	10,205.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	187,768.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	(b) (4)	(b) (4)	(b) (4)
		Total Indirect Costs	(b) (4)

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	302,306.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	302,306.00

L. Budget Justification

(Only attach one file.) 1234-Budget Justification.pdf

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		(b) (4)
Section B, Other Personnel		(b) (4)
Total Number Other Personnel	15	
Total Salary, Wages and Fringe Benefits (A+B)		437,310.00
Section C, Equipment		
Section D, Travel		7,087.00
1. Domestic	7,087.00	
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		90,279.00
1. Materials and Supplies	44,315.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	45,964.00	
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		534,676.00
Section H, Indirect Costs		(b) (4)
Section I, Total Direct and Indirect Costs (G + H)		860,827.00
Section J, Fee		
Section K, Total Costs and Fee (I + J)		860,827.00

Personnel

Heath Gasier, PhD, RD - Principal Investigator (2.4 calendar months effort in year 1, 4.8 calendar months effort in years 2 and 3; $(b) (4)). Dr. Gasier is an Assistant Professor in the Department of Anesthesiology, Duke University, and faculty within the Duke Center for Hyperbaric Medicine and Environmental Physiology (CHMEP). Dr. Gasier will be responsible for the overall administration and direction of the project, perform fluorescence and confocal imaging, data analyses, complete annual reports and presentations, and draft and or edit all manuscripts.

(b) (6) – Co-Investigator (2.4 calendar months effort in year 1, 4.8 calendar months effort in years 2 and 3; $(b) (4)). Dr. (b) (6) is an (b) (6) in the Department of Anesthesiology, Duke University, and faculty within the CHMEP. Dr. (b) (6) will be responsible for directing (b) (6). Dr. (b) (6) will perform the majority of the fluorescence and confocal imaging, analyze data, and assist with preparing reports and writing manuscripts. She will report to Dr. Gasier.

(b) (6) – Research Technician II (RT II) (0.12 calendar months effort in year 1, 0.18 calendar months effort in year 2, 0.06 calendar months in year 3; $(b) (4)). (b) (6) is a RT II within the CHMEP. He will be responsible for directing hyperbaric chamber operations. He will report to Dr. Gasier.

(b) (6) – Hyperbaric Chamber Specialist (HCS) (0.24 calendar months effort in year 1, 0.36 calendar months effort in year 2, 0.12 calendar months in year 3; $(b) (4)). (b) (6) is a HCS within the CHMEP. He will be responsible for performing the HBO_2 exposures. He will report to (b) (6) .

(b) (6) – LRA I (2.4 calendar months effort in year 1, 4.8 calendar months effort in year 2, 6 calendar months effort in year 3; $(b) (4)). (b) (6) is a LRA I within the CHEP. She will be responsible for purchasing, data management, tissue and blood collection, immunofluorescence, immunohistochemistry and immunoblotting. She will report to Dr. (b) (6) .

(b) (6) – Laboratory Research Analyst I (LRA I) (1.8 calendar months effort in year 1, 3.6 calendar months effort in year 2, 1.8 calendar months effort in year 3; $(b) (4)). (b) (6) is a LRA I within the Department of Anesthesiology, Duke University. She will be responsible for maintaining the mito-QC mice colony and caring for WT mice upon arrival from Jackson Laboratory. She will report to Dr. (b) (6)

Martha Salinas, BS – RT II (2.4 calendar months effort in year 1, 3.6 calendar months effort in years 2 and 3; $(b) (4)). (b) (6) within the CHEP. She will be responsible for monitoring the animals during HBO_2 exposures and recording seizure latencies, exercise training, immunofluorescence, immunohistochemistry and immunoblotting, and ELISAs. She will report to (b) (6) .

Materials and Supplies:

1. **Laboratory supplies**

   Year 1 ($5,340)

   - Gloves (Fisher Scientific, 19-13-1597B (small), 19-130-1597C (medium), 19-130-

1597D (large)), $350/case × 2 = $700
- Pipette tips (Fisher Scientific, 02-100-506 (10 µl), 02-100-507 (200 µl), 02-100-510 (1250 µl) $170 × 3 = $510
- Kimwipes (Fisher Scientific, 06-066), $150
- 1 ml syringes (Fisher Scientific, 22-253-260), $65 × 2 = $130
- Sterile PBS (Fisher Scientific, AAJ61196AP), $30
- 15 ml conical centrifuge tubes (Fisher Scientific, 14-955-238), $130
- 2 ml Eppendorf tubes (Fisher Scientific, 05-402-12), $105
- 1 ml blood collection microtubes (SAI, PMTP-H-1.3), $45
- Tiagabine hydrochloride (Sigma Aldrich, 1667280), $600
- PVDF membranes (VWR, 97062-900), $950
- Novex™ Tris-Glycine SDS Running Buffer (10X) (Fisher Scientific, LC2675), $35 × 2 = $70
- Novex™ WedgeWell™ 4 to 20%, Tris-Glycine, 1.0 mm, Mini Protein Gel, 15-well (Fisher Scientific, XPO4205BOX), $125 × 5 = $625
- Protease inhibitor (Fisher Scientific, PIA32963), $350
- RIPA Buffer (Fisher Scientific, AAJ61951AP), $115
- Hypodermic needles (Fisher Scientific, 14-826-48 (27g), 14-826-6C (23g)), $25 × 4 = $100
- Sterile saline (Fisher Scientific, 50-103-1363), $25 × 4 = $100
- Guaze (Fisher Scientific, NC9423256), $20 × 4 = $80
- Flouro-Jade C (VWR, 76262-660), $550

Year 2 ($2,605)
- Gloves (Fisher Scientific, 19-13-1597B (small), 19-130-1597C (medium), 19-130-1597D (large)), $350/case × 2 = $700
- Pipette tips (Fisher Scientific, 02-100-506 (10 µl), 02-100-507 (200 µl), 02-100-510 (1250 µl) $170 × 2 = $340
- Sterile PBS (Fisher Scientific, AAJ61196AP), $30
- Novex™ Tris-Glycine SDS Running Buffer (10X) (Fisher Scientific, LC2675), $35
- Novex™ WedgeWell™ 4 to 20%, Tris-Glycine, 1.0 mm, Mini Protein Gel, 15-well (Fisher Scientific, XPO4205BOX), $125 × 5 = $625
- 2 ml Eppendorf tubes (Fisher Scientific, 05-402-12), $105
- $30 × 2 = $60
- Parafilm (Fisher Scientific, 13-374-12), $160
- Flouro-Jade C (VWR, 76262-660), $550

2. Assays

Year 1 ($18,260)
- Progesterone ELISA (LSBio, LS-F10020), $800 × 6 = $4800
- Estrogen ELISA (LSBio, LS-F39304), $800 × 6 = $4800

- Norephinephrine (Rocky Mountain Diagnostics, Inc., BAE-5200R), $650 × 10 = $6500
- Tunnel Assay (Abcam, ab206386) = $720 × 3 = $2160

Year 2 ($6,400)

- S100B ELISA (LSBio, LF-5980-1) = $800 × 8 = $6400

3. **Antibodies ($7,610 in Year 2)**
    - Anti-GABA (Sigma-Aldrich, ABN131), $410 × 2 = $820
    - Anti-GABA Transporter 1 (Abcam, ab259971), $600 × 2 = $1200
    - IHC polymer detection kit (Abcam, ab209101), $200 × 3 = $600
    - Goat anti-rabbit IgG H&L (Abcam, ab150077), $150 × 2 = $300
    - Anti-glutamic acid decarboxylase 65 (Sigma-Aldrich, G4913), $650 × 2 = 1300
    - Anti-glutamic acid decarboxylase 67 (Sigma-Aldrich, G5419), $500 × 2 = $1000
    - Anti-glutaminase (ThermoFisher Scientific, MA5-41171), $420 × =$840
    - Anti-aromatase (ThermoFisher Scientific, BS-1292R), $350
    - Anti-GFAP (Abcam, ab7260), $600 × 2 = $1200

4. **Accuris SmartWasher 96, Microplate Washer** (Southern Labware, MW9600), $3,800 in Year 2
    - Manifolds for Accuris SmartWasher 96 (Southern Labware, MW9600-ACC), $300 in Year 2

Other:

- Breeding mito-QC homozygotes ((b) (6) [redacted]), $15,000 ($5,000 in year 1 and $10,000 in year 2)
- C57BL/6 mice (Jackson Laboratory), $5,000 ($2,300 in year 1 and $2,700 in year 2)
- Per diem (Duke Division of Laboratory Animal Resources), $9,500 ($1,500 in year 1 and $8,000 in year 2)
- UPLC-MS/MS GABA/glutamine/glutamate (Duke Proteomics and Metabolomics Core Services), $9,000 in year 1
- Process/Embed in Paraffin, H&E stain, serial section (Duke University Substrate Services Core & Research Support), $6,264 ($1,740 in year 1 and $4,524 in year 2)
- Confocal microscopy, $1,200 in year 3

Travel:

- ONR Program Review
    - Justification: The purpose of these trips is for the PI (one traveler) to present the data from the project. Estimated costs were obtained from US GSA.
        - 2023 (assuming 5% increase)
            - Airfare: $677
            - Lodging: $260 x 4 = $1092
            - M&IE: $79 x 4 = $332
            - Taxi to and from airport: $53
            - Total = $2154

        - 2024 (assuming 5% increase)
            - Airfare: $711
            - Lodging: $260 x 4 = $1147
            - M&IE: $79 x 4 = $350
            - Taxi to and from airport: $56
            - Total = $2264

- Military Health System Research Symposium
    - Justification: The purpose of these trips is for the PI (one traveler) to present the data from the project. Estimated costs were obtained from US GSA.
    - 2023 (assuming 5% increase)
        - Airfare: $168
        - Lodging: $135 x 4 = $540
        - M&IE: $135 x 4 = $540
        - Taxi to and from airport: $53
        - Total = $1301

    - 2024 (assuming 5% increase)
        - Airfare: $176
        - Lodging: $142 x 4 = $568
        - M&IE: $142 x 4 = $568
        - Taxi to and from airport: $56
        - Total = $1368

COLLEGES AND UNIVERSITIES RATE AGREEMENT

EIN: 1560532129A1

ORGANIZATION:

Duke University
American Tobacco Campus
Box Number 104137
Durham, NC 27708

DATE:03/23/2021

FILING REF.: The preceding agreement was dated 09/17/2020

The rates approved in this agreement are for use on grants, contracts and other agreements with the Federal Government, subject to the conditions in Section III.

SECTION I: INDIRECT COST RATES

RATE TYPES: FIXED FINAL PROV. (PROVISIONAL) PRED. (PREDETERMINED)

EFFECTIVE PERIOD

TYPE	FROM	TO	RATE(%)	LOCATION	APPLICABLE TO
PRED.	07/01/2021	06/30/2023	(b) (4) [redacted]	On-Campus	Organized Research (A)
PRED.	07/01/2021	06/30/2023	[redacted]	Off-Campus	Organized Research (B)(C)
PRED.	07/01/2021	06/30/2023	[redacted]	Off-Campus	Organized Research (D)
PRED.	07/01/2021	06/30/2023	[redacted]	On-Campus	Instruction
PRED.	07/01/2021	06/30/2023	[redacted]	On-Campus	Other Sponsored Activities
PROV.	07/01/2023	Until Amended			Use same rates and conditions as those cited for fiscal year ending June 30, 2023.

*BASE

Modified total direct costs, consisting of all direct salaries and wages, applicable fringe benefits, materials and supplies, services, travel and up to the first $25,000 of each subaward (regardless of the period of performance of the subawards under the award). Modified total direct costs shall exclude equipment, capital expenditures, charges for patient care, rental costs, tuition remission, scholarships and fellowships, participant support costs and the portion of each subaward in excess of $25,000. Other items may only be excluded when necessary to avoid a serious inequity in the distribution of indirect costs, and with the approval of the cognizant agency for indirect costs.

SECTION I: FRINGE BENEFIT RATES**

TYPE	FROM	TO	RATE(%)	LOCATION	APPLICABLE TO
FIXED	7/1/2021	6/30/2022	(b) (4)	All	Exempt
FIXED	7/1/2021	6/30/2022	[redacted]	Medical Ctr.	House Staff / PostDocs
FIXED	7/1/2021	6/30/2022	[redacted]	All	Non-Exempt
FIXED	7/1/2021	6/30/2022	[redacted]	All	Temporary Services
FIXED	7/1/2021	6/30/2022	[redacted]	All	Students Taxable
FIXED	7/1/2021	6/30/2022	[redacted]	All	Ph.D. Students
PROV.	7/1/2022	Until amended			Use same rates and conditions as those cited for fiscal year ending June 30, 2022.

** DESCRIPTION OF FRINGE BENEFITS RATE BASE:

Salaries and wages.

SECTION II: SPECIAL REMARKS

TREATMENT OF FRINGE BENEFITS:

The fringe benefits are charged using the rate(s) listed in the Fringe Benefits Section of this Agreement. The fringe benefits included in the rate(s) are listed below.

TREATMENT OF PAID ABSENCES

Vacation, holiday, sick leave pay and other paid absences are included in salaries and wages and are claimed on grants, contracts and other agreements as part of the normal cost for salaries and wages. Separate claims are not made for the cost of these paid absences.

OFF-CAMPUS DEFINITION: The off-campus rate will apply for all activities: a) Performed in facilities not owned by the institution and where these facility costs are not included in the F&A pools; or b) Where rent is directly allocated/charged to the project(s). Grants or contracts will not be subject to more than one F&A cost rate. If more than 50% of a project is performed off-campus, the off-campus rate will apply to the entire project.

Fringe Benefits include: FICA, Retirement, Medical Care, Disability Insurance, Group Life Insurance, Survivor's Benefits, Fringe Benefits Administration, Child Care, Workers' Compensation and Unemployment Insurance.

Equipment means an article of nonexpendable tangible personal property having a useful life of more than one year, and an acquisition cost of $5,000 or more per unit.

APPLICATION OF INDIRECT COST RATES TO DOD CONTRACTS/SUBCONTRACTS:

In accordance with DFARS 231.303, no limitation (unless waived by the institution) may be placed on the reimbursement of otherwise allowable indirect cost rates incurred by an institution of higher education under a DOD contract awarded on or after November 30, 1993, unless the same limitation is applied uniformly to all other organizations performing similar work. It has been determined by the Department of Defense that such limitation is not being uniformly applied. Accordingly, the following rates do not reflect the application of the 26% limitation on administrative indirect costs imposed by OMB Circular A-21.

PRED. 07/01/21 to 06/30/23 (b)(4)% On-Campus Orgn. Rsch. (A)
PRED. 07/01/21 to 06/30/23 [redacted]% Off-Campus Orgn. Rsch. (B)(C)
PRED. 07/01/21 to 06/30/23 [redacted]% Off-Campus Orgn. Rsch. (D)
PROV. 07/01/23 Until Amended Use same rates and conditions as those cited for fiscal year ended June 30, 2023.

Next Fringe Benefit rates proposal for fiscal year ending 06/30/2021 is due in our office by 12/31/2021. Next indirect cost rate proposal for fiscal year ending 6/30/2022 is due in our office by 12/31/2022.

This rate agreement updates the Fringe Benefits section only.

SECTION III: GENERAL

A. LIMITATIONS:

The rates in this Agreement are subject to any statutory or administrative limitations and apply to a given grant, contract or other agreement only to the extent that funds are available. Acceptance of the rates is subject to the following conditions: (1) Only costs incurred by the organization were included in its facilities and administrative cost pools as finally accepted: such costs are legal obligations of the organization and are allowable under the governing cost principles; (2) The same costs that have been treated as facilities and administrative costs are not claimed as direct costs; (3) Similar types of costs have been accorded consistent accounting treatment; and (4) The information provided by the organization which was used to establish the rates is not later found to be materially incomplete or inaccurate by the Federal Government. In such situations the rate(s) would be subject to renegotiation at the discretion of the Federal Government.

B. ACCOUNTING CHANGES:

This Agreement is based on the accounting system purported by the organization to be in effect during the Agreement period. Changes to the method of accounting for costs which affect the amount of reimbursement resulting from the use of this Agreement require prior approval of the authorized representative of the cognizant agency. Such changes include, but are not limited to, changes in the charging of a particular type of cost from facilities and administrative to direct. Failure to obtain approval may result in cost disallowances.

C. FIXED RATES:

If a fixed rate is in this Agreement, it is based on an estimate of the costs for the period covered by the rate. When the actual costs for this period are determined, an adjustment will be made to a rate of a future year(s) to compensate for the difference between the costs used to establish the fixed rate and actual costs.

D. USE BY OTHER FEDERAL AGENCIES:

The rates in this Agreement were approved in accordance with the authority in Title 2 of the Code of Federal Regulations, Part 200 (2 CFR 200), and should be applied to grants, contracts and other agreements covered by 2 CFR 200, subject to any limitations in A above. The organization may provide copies of the Agreement to other Federal Agencies to give them early notification of the Agreement.

E. OTHER:

If any Federal contract, grant or other agreement is reimbursing facilities and administrative costs by a means other than the approved rate(s) in this Agreement, the organization should (1) credit such costs to the affected programs, and (2) apply the approved rate(s) to the appropriate base to identify the proper amount of facilities and administrative costs allocable to these programs.

BY THE INSTITUTION:

Duke University

(INSTITUTION)

(b) (6) [redacted]

(SIGNATURE)

(b) (6) [redacted]

(NAME)

AVP Finance

(TITLE)

3/25/2021

(DATE)

ON BEHALF OF THE FEDERAL GOVERNMENT:

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Darryl W. Mayes - S

Digitally signed by Darryl W. Mayes -S
DN: c=US, o=U.S. Government, ou=HHS, ou=PSC, ou=People, 0.9.2342.19200300.100.1.1=2000131669, cn=Darryl W. Mayes -S
Date: 2021.03.24 09:40:11 -04'00'

(SIGNATURE)

Darryl W. Mayes

(NAME)

Deputy Director, Cost Allocation Services

(TITLE)

3/23/2021

(DATE) 2783

HHS REPRESENTATIVE: Lucy Siow

Telephone: (301) 492-4855

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: (b) (6) [redacted]

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6) [redacted]

POSITION TITLE: (b) (6) [redacted]

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
(b) (6) [redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

(b) (4) [redacted]

(b) (4), (b) (6) [redacted]

B. Positions, Scientific Appointments, and Honors

(b) (6)

(b) (6)

(b) (6)

(b) (4)

(b) (4), (b) (6)

(b) (4), (b) (6)

(b) (4), (b) (6)

(b) (4)

(b) (4), (b) (6)

(b) (6)

OTHER SUPPORT

(b) (6) [redacted]

POSITIONS AND APPOINTMENTS
2009 – Present: Associate Professor, Anesthesiology, Duke University Medical Center
2010 – Present: Faculty Member, Duke Cancer Center
2010 – Present: Faculty Member, Duke Graduate School

IN-KIND
None

PREVIOUS (within the past 5 years)

Title: Redox Regulation of Lung Mitochondrial Biogenesis in Sepsis/Pneumonia (4R01-AI095424-05)
Time Commitment: 48% 5.76 calendar months Co-Investigator
Supporting Agency: NIH
Name/Address of Funding Agency's Procuring Contracting/Grants Officer:
Grants Management Specialist: Carine Normil
Phone: 301-496-7075
Email: carine.normil@nih.gov
Program Official: Wendy Davidson
Phone: 240-627-3494
Email: davidsonw@mail.nih.gov
Performance Period: 07/01/2012-06/30/2017
Level of Funding: $250,000 (Annual Directs); $392,500 (Total Costs)
Brief Description of Projects Goals: We propose that the program of mitochondrial biogenesis mediates lung protection through HO-1/CO activation of Nfe2l2 and NRF-1 leading to 1) anti-inflammatory Socs3 and IL10 gene expression, 2) suppression of inflammasome-mediated IL-1 production, and 3) activation of mitophagy through Bnip3 and Atg5, promoting alveolar epithelial cell survival and resolution of barrier dysfunction. Our Specific Aims are: Aim 1: Determine whether Nfe2l2 and NRF1 induction of lung mitochondrial biogenesis in murine S. aureus sepsis and pneumonia up-regulates Socs3 and Il10 anti-inflammatory gene expression, suppresses caspase1 cleavage and IL-1 production and mitigates lung inflammation and ALI. Aim 2: Use gain and loss of function studies to determine whether Nfe2l2 and NRF1 induction of lung of mitochondrial biogenesis a) regulates the autophagy genes Bnip3 and Atg5 and b) activates pro- survival mitophagy through HO-1/CO-related mitochondrial ROS generation in murine S. aureus pneumonia. Aim 3: Assess the extent, location, and relationship of mitochondrial biogenesis to mitophagy in the alveolar epithelium of human ALI/ARDS patients compared with healthy human lung.

Title: Project 2: Regulation of Mitochondrial Quality Control by Heme Oxygenase-1 System in Sepsis (107287)
Time Commitment: 50% 6.0 calendar months Co-Investigator
Supporting Agency:
Name/Address of Funding Agency's Procuring Contracting/Grants Officer:
(b) (6) [redacted]
Research Administration and Support
(b) (6) [redacted]
Email: (b) (6) [redacted]
Phone: (b) (6) [redacted]
Performance Period: 08/15/2011-06/30/2017
Level of Funding: $276,743 (Annual Directs); $1,600,971 (Total Costs)

Brief Description of Projects Goals: The purpose of this project is to study the effects of low-level carbon monoxide on mitochondrial biogenesis.

ACTIVE

Title: Mitochondrial quality control and alveolar damage resolution after acute lung injury (5R01-HL135239-04)
Time Commitment: 50% 6 calendar months Co-Investigator
Supporting Agency: NIH
Name/Address of Funding Agency's Procuring Contracting/Grants Officer:
Grants Management Specialist: Kevin Heath
Phone: 301-827-8033
Email: heathkj@mail.nih.gov
Program Official: Guofei Zhou
Phone: 301-435-0222
Email: guofei.zhou@nih.gov
Performance Period: 08/10/2017-06/30/2022
Level of Funding: $250,000 (Annual Directs); $1,590,000 (Total Costs)
Brief Description of Projects Goals: We propose that the AT2 cell mitochondrial QC program is necessary for AT1 proliferation and the full resolution of alveolar epithelial damage in ALI. This idea is not just novel; it is amenable to therapeutic intervention. Thus, targeted pharmacological interventions may shorten the course of ARDS. Therefore, using molecular methods with a preclinical approach in mice and cells, we will rigorously test transcriptional activation of mitochondrial QC in the AT2 cell as a critical factor in ALI resolution through enablement of AT2 cell proliferation, trans-differentiation into AT1 cells, and control of inflammation, all of which are necessary to restore epithelial barrier function. We will test our novel ideas with three Specific Aims: Aim 1) Measure and localize oxidant damage in lung parenchyma and AT2 cell mitochondria (specifically mtDNA oxidation) in ALI/pneumonia in mice and its impact on mitochondrial QC regulation by HO-1/CO and a) AT2 cell apoptosis, b) resolution of alveolar inflammation and c) alveolar barrier dysfunction. Aim 2) Test how HO-1 induction of the mitochondrial QC network a) activates mitophagy b) accelerates AT2 cell proliferation and transdifferentiation into type I epithelium, and c) prevents necroptosis after ALI/pneumonia. Aim 3) Demonstrate whether strategies that activate mitochondrial QC will reverse loss of lung protection in ALI/pneumonia in mice with conditional knockout (CKO) of a) HO-1 or b) NRF-1 in AT2 cells using the approaches of Aim 1.

Title: Effects of HBO2 on skeletal muscle calcium regulation and mitochondrial function (N00014-21-1-2279)
Time Commitment: 15% 1.8 calendar months Co-Investigator
Supporting Agency: Office of Naval Research (ONR)
Name/Address of Funding Agency's Procuring Contracting/Grants Officer:
Technical Specialist: Sandra Chapman
Email: Sandra.chapman.ctr@navy.mil
Contract Specialist: Veronica Y. Lacey
Email: veronica.lacey@navy.mil
Performance Period: 04/22/2021-04/23/2022
Level of Funding: $173,939 (Annual Directs); $563,616 (Total Costs)
Brief Description of Projects Goals: The long-term goal is to identify new approaches for preventing oxygen toxicity and optimizing performance in divers. The objective is determine the impact of skeletal muscle oxidant production on calcium regulation and mitochondrial function in mice exposed to HBO2.

PENDING

Title: Novel mitochondrial protective properties of annexin A1 (1R01-DK131065-01)
Time Commitment: 10% 1.2 calendar months Co-Investigator
Supporting Agency: NIH
Name/Address of Funding Agency's Procuring Contracting/Grants Officer:

Grants Management Specialist: Norma G. DeGuzman
Phone: 301-480-1810
Email: norma.deguzman@nih.gov
Program Official: Anna Burkart Sadusky
Phone: 301-827-7036
Email: anna.sudusky@nih.gov
Performance Period: 12/01/2021-11/30/2026
Level of Funding: $200,000 (Annual Directs); $1,610,000 (Total Costs)
Brief Description of Projects Goals: Acute kidney injury (AKI) is a common complication in post-surgical patients, and it significantly decreases patient quality of life and leads to increased rates of death. The proposed research in this grant application is relevant to public health because it seeks to understand the cellular mechanism of a novel kidney protective therapeutic that could be given prior to surgery or kidney transplantation to protect the kidney from injury. Thus, this proposal fulfills both aspects of the NIH's mission: to seek fundamental knowledge about the nature and behavior of living systems and the application of that knowledge to enhance health, lengthen life, and reduce illness and disability.

OVERLAP

There is no scientific, budgetary or effort overlap.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

Signature: _____

Date: _____

SPOC ¯ 09/21/2021 ¯ (b) (6) [redacted] (Anesthesiology)

Reviewed ¯ 09/21/2021 ¯ (b) (6) [redacted] (Anesthesiology)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Gasier, Heath

eRA COMMONS USER NAME (credential, e.g., agency login): hgasier

POSITION TITLE: Assistant Professor, Department of Anesthesiology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Youngstown State University, OH	B.S.A.S.	06/2000	Food & Nutrition-Dietetics
University Hospitals of Cleveland, OH	Internship	08/2001	Dietetics
Case Western Reserve University, OH	M.S.	01/2001	Nutrition
Texas A&M University, TX	Ph.D.	06/2009	Exercise Physiology
Duke University School of Medicine, NC	Postdoctoral	05/2015	Hyperbaric Physiology

A. Personal Statement

My role in the proposed project is that of a PI. I have a broad background in nutrition and physiology, and have worked as a U.S. Air Force Clinical Dietitian for three years in between my graduate education. During my time as a clinician, I became interested in metabolic changes in the critically ill and aged patients because I often observed rapid deterioration of their health and function. This experience led me back to graduate school where I directed my studies on skeletal muscle metabolism with a focus on anabolism. I was supported by a U.S Navy Health Services Collegiate Program Scholarship and returned to uniform upon graduation. For the next decade, I served in the U.S. Navy as a Research Physiologist and conducted numerous projects as a PI/Co-I prior to retiring in 2019. The research areas include bone health in specific military occupations, obesity, central nervous system and pulmonary oxygen toxicity, hypobaric hypoxia, and skeletal muscle mitochondrial dynamic regulation in heat stress and obesity. I have conducted investigations using cells, rodents, fish and human models. I have led large-scale human studies in the laboratory and field. I have mentored physicians, undergraduate and graduate students, and research technicians. While my faculty rank is Assistant Professor, my positions and experiences in the military and as a scientist have provided me with the knowledge and skills of a more senior researcher. I understand the importance of constructing a realistic research plan, timeline and budget. I am aware of the benefit of frequent communication among all research team members, and have previously worked with the Co-I's and research technicians. I believe that my documented success in procuring research funding, directing, meeting deadlines, and accurate reporting of study results provides evidence that I am fully qualified to carry out this research as a PI.

B. Positions, Scientific Appointments, and Honors

Positions

2019- Assistant Professor, Department of Anesthesiology, Duke University Center for Hyperbaric Medicine & Environmental Physiology, Durham, NC

2015-2019 Assistant Professor, Department of Military & Emergency Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

2013-2015 Visiting Research Scholar, Duke University, Center for Hyperbaric Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

2009-2013 Research Physiologist, Naval Submarine Medical Research Laboratory, Groton, CT

2002-2005 Clinical Dietitian, Wilford Hall Medical Center, Lackland Air Force Base, San Antonio, TX
2005-2009 Graduate Research Assistant, Texas A&M University, Department of Health and Kinesiology, College Station, TX

Scientific Appointments

2015-2019 Navy Appointee, Nutrition and Dietary Supplements Working Group, Joint Program Committee-5/Military Operational Medicine Research Program
2015-2019 IACUC Alternate, Department of Military & Emergency Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD
2015-2019 Scientific Advisor for Animal Research, Consortium for Health and Military Performance, Department of Military & Emergency Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD
2010-2013 Vice-Chair Scientific Review Board, Naval Submarine Medical Research Laboratory, Groton, CT
2009-2013 IRB Alternate, Naval Submarine Medical Research Laboratory, Groton, CT
2009-2013 Research Integrity Leader, Naval Submarine Medical Research Laboratory, Groton, CT

Honors

2013-2015 Hyperbaric Physiology Fellowship (Duke Center for Hyperbaric Medicine and Environmental Physiology, Durham, NC), United States Navy
2005-2009 Health Services Collegiate Program Scholarship (Texas A&M University, College Station, TX), United States Navy
1999 Outstanding Member, Golden Key National Honor Society, Youngstown State University, Youngstown, OH

C. Contributions to Science

1. **Assessing Skeletal Muscle Protein Synthesis using ^2H_2O: An Alternative Approach.** Researchers previously relied on labeled amino acids to measure protein renewal rates. This methodology was constrained by the need to confine human subjects to a laboratory for catheter placement that was used for continuous isotope infusion and blood sampling. In addition, subjects were required to report in the laboratory after an overnight fast and movement was limited to a bout of exercise that would be performed after a certain period of isotope infusion. My doctorate advisor, Dr. James Fluckey, and I questioned this well established and relied upon methodology because it clearly did not reflect free-living conditions, e.g. habitual food intake, activities of daily living, sleep, stress, circadian rhythm. In collaboration with Dr. Steven Previs, we performed a series of experiments in rats, fish and humans using deuterium oxide (^2H_2O) and GC-MS technology. We determined that skeletal muscle protein renewal could be determined over short- or long-time intervals without the need for laboratory confinement. This method is now routinely used by researchers from various disciplines to measure skeletal muscle protein anabolism under free-living conditions over extended periods.
   a. Gasier HG, Fluckey JD, Previs SF, Wiggs MP, Riechman SE. Acute resistance exercise augments integrative myofibrillar protein synthesis. Metabolism. 2012 Feb;61(2):153-156. PMID: 21864869
   b. Gasier HG, Riechman SE, Wiggs MP, Buentello A, Previs SF, Fluckey JD. Cumulative responses of muscle protein synthesis are augmented with chronic resistance exercise training. Acta Physiol (Oxf). 2011 Mar;201(3):381-389. PMID: 20804462
   c. Gasier HG, Previs SF, Pohlenz C, Fluckey JD, Gatlin DM 3rd, Buentello JA. A novel approach for assessing protein synthesis in channel catfish, Ictalurus punctatus. Comp Biochem Physiol B Biochem Mol Biol. 2009 Oct;154(2):235-238. PMID: 19563906
   d. Gasier HG, Riechman SE, Wiggs MP, Previs SF, Fluckey JD. A comparison of ^2H_2O and phenylalanine flooding dose to investigate muscle protein synthesis with acute exercise in rats. Am J Physiol Endocrinol Metab. 2009 Jul;297(1):E252-259. PMID: 19366878

2. **Impact of Submergence on Bone and Metabolic Health.** Prolonged submergence imposes stress on human biology because there is no sunlight, limited space and equipment to perform regular exercise, shift work and the option to consume energy dense food. As the PI, I led two large-scale investigations to determine the impact of submarine duty on short- (1 submarine patrol) and long-term (course of a career) skeletal health. A secondary objective was to measure body composition and metabolic health. From this body of work, we learned that submarine duty does not lead to a greater than normal age-related decline in

bone density. Despite this finding, approximately 5% of submariners >50 years of age had osteoporosis and 12% met the FRAX criteria for pharmacological treatment. We also determined that > 2/3 of submariners had a body fat percentage diagnostic of obesity (>25%) when measured by dual-energy X-ray absorptiometry or air displacement plethysmography. In addition, the prevalence of metabolic syndrome in submariners was similar to the general population (age and gender matched), a finding that was alarming since the military has higher health and fitness standards. This research was important because it established that submarine patrols do not cause bone loss but contributes to obesity and metabolic syndrome.

a. Gasier HG, Young CR, Gaffney-Stomberg E, McAdams DC, Lutz LJ, McClung JP. Cardiometabolic Health in Submariners Returning from a 3-Month Patrol. Nutrients. 2016 Feb 9;8(2):85. PMID: 26867201
b. Gasier HG, Hughes LM, Young CR, Richardson AM. Comparison of Body Composition Assessed by Dual-Energy X-Ray Absorptiometry and BMI in Current and Former U.S. Navy Service Members. PLoS One. 2015;10(7):e0132157. PMID: 26197480
c. Gasier HG, Hughes LM, Young CR, Richardson AM. The assessment of bone mineral content and density of the lumbar spine and proximal femur in US submariners. Osteoporos Int. 2014 Sep;25(9):2225-2234. PMID: 24866394
d. Gasier HG, Gaffney-Stomberg E, Young CR, McAdams DC, Lutz LJ, McClung JP. The efficacy of vitamin D supplementation during a prolonged submarine patrol. Calcif Tissue Int. 2014 Sep;95(3):229-239. PMID: 25005834

3. **Mitigation of Central Nervous System Oxygen Toxicity.** The toxic effects of oxygen on the central nervous system (CNS) were first reported in 1878 by Paul Bert. Lorrain Smith later (1899) determined that oxygen was also toxic to the lung. While assigned to the Naval Submarine Medical Research Laboratory, Groton, CT, I became fascinated in how hyperbaric oxygen (HBO_2) could be used to treat decompression illness and many other complications but was also toxic to the CNS and lungs. During and after the completion of a two-year fellowship in hyperbaric physiology at the Duke Center for Hyperbaric Medicine and Environmental Physiology, I (Co-I) completed a serious of experiments with Dr. Claude Piantadosi and Dr. Ivan Demchenko that led to significant advancements in the field: First, there are regional neurovascular responses to HBO_2, and the non-selective beta-antagonist propranolol can effectively delay cerebral hyperemia and seizure onset in rats. Second, GABA production is reduced, and this is in part modulated by S-nitrosylation of GAD65. Third, the combination of FDA approved antiepileptic drugs from different functional classes (GABA enhancers and Na+-channel antagonists) offer greater antiseizure protection in HBO_2 than individual antiepileptic drugs. This research was novel because it led to the discovery of effective pharmacological approaches that can delay oxygen-induced seizures and attenuate acute lung injury.

a. Demchenko IT, Zhilyaev SY, Alekseeva OS, Krivchenko AI, Piantadosi CA, Gasier HG. Increased antiseizure effectiveness with tiagabine combined with sodium channel antagonists in mice exposed to Hyperbaric Oxygen. Neurotox Res. 2019 Nov;36(4):788-795. PubMed PMID: 31148118
b. Gasier HG, Demchenko IT, Zhilyaev SY, Moskvin AN, Krivchenko AI, Piantadosi CA. Adrenoceptor blockade modifies regional cerebral blood flow responses to hyperbaric hyperoxia: Protection against CNS oxygen toxicity. J Appl Physiol. 2018 Jul;125:1296-1304. PubMed PMID: 30024340
c. Gasier HG, Demchenko IT, Tatro LG, Piantadosi CA. S-nitrosylation of GAD65 is implicated in decreased GAD activity and oxygen-induced seizures. Neurosci Lett. 2017 Jul 13;653:283-287. PubMed PMID: 28579483
d. Gasier HG, Demchenko IT, Allen BW, Piantadosi CA. Effects of striatal nitric oxide production on regional cerebral blood flow and seizure development in rats exposed to extreme hyperoxia. J Appl Physiol (1985). 2015 Dec 1;119(11):1282-1288. PubMed PMID: 26338456

4. **Induction of HO-1/CO to Prevent Diet-Induced Obesity Metabolic Sequela.** Heme oxygenase (HO)-1 catalyzes the breakdown of heme-iron in the presence of oxygen and NADPH generating carbon monoxide (CO), iron and biliverdin. CO is a cell signaling molecule that activates antioxidant and anti-inflammatory responses to stress while also regulating mitochondrial quality control (dynamics, mitophagy and biogenesis). Because oxidant production and inflammation are increased, and mitochondrial quality control is altered in obesity, my research team tested CO's anti-obesity potential using in vitro and in vivo rodent models. After establishing an in vitro model to study obesity and diabetes with Dr. Tianzheng Yu, we learned the following: First, CO prevents nutrient excess induced mitochondrial fission by repressing Drp1 recruitment to mitochondria, favoring fusion and increased mitochondrial respiration. Second, CO and exercise training prevents obesity and the metabolic sequela during chronic overfeeding and increases

resting energy expenditure. Third, low-dose inhaled CO does not negatively impact bone microarchitecture and strength during periods of nutrient excess. This work is innovative because the results define a mechanism for CO's therapeutic potential in diet-induced obesity, and highlight its safety on the skeleton.

a. Gasier HG, Dohl J, Suliman HB, Piantadosi CA, Yu T. Skeletal muscle mitochondrial fragmentation and impaired bioenergetics from nutrient overload is prevented by carbon monoxide. Am J Physiol Cell Physiol. 2020 Oct;319(4):C746-756. PMID: 32845721
b. Gasier HG, Yu T, Swift JM, Metzger CE, McNerny EM, Swallow EA, Piantadosi CA, Allen MR. Carbon Monoxide and Exercise Prevents Diet-Induced Obesity and Metabolic Dysregulation Without Affecting Bone. Obesity (Silver Spring). 2020 May;28(5):924-931. PMID: 32237119
c. Dohl J, Foldi J, Heller J, Gasier HG, Deuster PA, Yu T. Acclimation of C_2C_{12} myoblasts to physiological glucose concentrations for in vitro diabetes research. Life Sci. 2018 Oct;211:238-244. PMID: 30253137

Complete List of Published Work in My Bibliography:
https://www.ncbi.nlm.nih.gov/myncbi/heath.gasier.1/bibliography/public/

OTHER SUPPORT

Gasier, Heath

CURRENT POSITIONS & APPOINTMENTS

2019- Assistant Professor, Department of Anesthesiology, Duke University Center for Hyperbaric Medicine & Environmental Physiology, Durham, NC

IN-KIND

None

ACTIVE:

Title: Effects of HBO2 on skeletal muscle calcium regulation and mitochondrial function (N00014-21-1-2279)
Time commitment: 40% 4.8 calendar months
Supporting agency: Office of Naval Research
One Liberty Center
875 N. Randolph Street, Suite 1425 Arlington, VA
Program Officer: Sandra Chapman (sandra.chapman@navy.mil) Period of performance: 2021-2022
Funding level: $144,091 (Directs); $227,954 (Total costs)
Project goal: To identify new approaches for preventing oxygen toxicity and optimizing performance in divers.
Specific aims: 1) Measure skeletal muscle oxidant production in C57BL/6 mice exposed to three session of HBO2 at 2.5 atmospheres absolute (ATA) or air for 6 h. 2) Measure skeletal muscle oxidation of RyR1 and STIM1, and calcium responses in C57BL/6 mice exposed to three sessions of HBO2 at 2.5 atmospheres (ATA) or air for 6 h. 3) Measure skeletal muscle mitochondrial membrane potential ($\Delta\Psi m$) and cellular energy state in C57BL/6 mice exposed to three session of HBO2 at 2.5 atmospheres absolute (ATA) or air for 6 h.
Project overlap: No overlap
Role: PI

Title: Critical molecular targets for the toxic effects of HBO_2 and novel pharmacological approach for preventing acute toxicity (N00014-18-1-2702)
Time commitment: 0% (NCE/effort pending)
Supporting agency: Office of Naval Research
One Liberty Center
875 N. Randolph Street, Suite 1425Arlington, VA
Program Officer: Sandra Chapman (sandra.chapman@navy.mil)
Period of performance: 2018 – 2022
Funding level: $492,971 (Directs); $988,057 (Total costs)
Project goal: To understand and compare how antiepileptic drug combinations influences sub-cellular oxidative and nitrosative attack in the brains and lungs of mice to standardize schedules of HBO_2.
Specific aims: 1) Measure oxidative tissue and mitochondrial damage by 5 ATA HBO_2 in mouse forebrain including hippocampus and hindbrain using standard biochemical tests under conditions of A) Control, B) HBO_2, C) AED combinations alone, and D) HBO_2 plus AED combinations. 2) Measure basic neurotransmitter levels in mouse forebrain including hippocampus and hindbrain under conditions of A) Control, B) HBO_2, C) AED combinations only, and D) HBO_2 plus AED combinations. 3) Measure inflammatory activity in mouse forebrain and hindbrain, lung parenchyma, and mouse plasma and compare the four conditions outlined in Aims 1 and 2. 4) Measure cell death at days 1 and 7 in the brain and compare it with changes in motor coordination by parallel bar.
Project overlap: No overlap
Role: Co-investigator from 2018-2020 (PI Claude Piantadosi), PI 2020-2021

Title: Heme oxygenase-1 regulation of skeletal muscle inflammation and mitochondrial fitness in sarcopenic obesity
Time commitment: 0% 0 calendar months
Supporting agency: DREAM Innovation Grant
Department of AnesthesiologyDuke University
5692 HAFS Bldg., DUMC 3094
Durham, NC 27710
Administrative contact: Sandra C. Yee-Benedetto (sandra.yee.benedetto@duke.edu)
Period of performance: 2020 – 2021
Funding level: $26,087 (Directs); $30,000 (Total costs)
Project goal: To find new strategies for preserving skeletal muscle mass and metabolic health in obesity and aging.
Specific aims: 1) Determine the impact of sarcopenic obesity on the skeletal muscle HO-1 enzyme system. 2) Evaluate the role of skeletal muscle HO-1 induction on oxidation production, inflammation and mitochondrial quality control in sarcopenic obesity.
Role: PI

Title: Perfluoromethane to reduce decompression sickness (N00024-21-C-4310)
Time commitment: 5% 0.6 calendar months
Supporting agency: Naval Sea Systems Command

1333 Isaac Hull Ave. SEWashington, DC
20376
Program Officer: DBSD (202-781-1003)
Period of performance: 2021 – 2023
Funding level: $218,682 (Directs); $361,919 (Total costs)
Project goal: Evaluate the efficacy of perfluoromethane on reducing decompression sickness in awake pigs.
Specific aims: 1) Determine if changing the breathing gas to perfluoromethane before decompression from 200 fsw can reduce the onset of decompression sickness.
Project overlap: No overlap
Role: Co-investigator (PI Richard Moon)

PENDING

Title: Ketone ester supplement for central nervous system oxygen toxicity (CNSOT) mitigation
Time commitment: 10% 1.2 calendar months
Supporting agency: Naval Sea Systems Command
1333 Isaac Hull Ave. SEWashington, DC
20376

Program Officer: DBSD (202-781-1003)
Period of performance: 2022 – 2024
Funding level: $825,248 (Total costs)
Project goal: Evaluate a ketone diester (KDE) for delaying or preventing the onset of CNS oxygen toxicity in the working diver while also evaluating the effects of KDE supplementation and elevated oxygen partial pressure (PO2) on cognition, and physiologic variables, increasing our overall scientific knowledge of CNS O2 toxicity.
Specific aims: 1) Evaluate the rise in serum ketone levels (beta-hydroxybutyrate, acetoacetate and acetone) after consumption of ketone diester supplement. 2) Select an optimal ketone diester supplement strategy based on detected serum ketone levels and subject tolerability. 3) Determineif CNSOT can be delayed or prevented by ketone diester supplementation. 4 Evaluate the impactof ketone ester supplementation on cognitive performance at sea level and 2.06 ATA breathing 100% O2. 5) Investigate the physiologic factors associated with the onset of CNS O2 toxicity.
Project overlap: No overlap
Role: Co-Investigator (PI Bruce Derrick)

Title: Metabolic system for in vivo rodent testing

Time commitment: 0%
Supporting agency: Office of Naval Research
One Liberty Center
875 N. Randolph Street, Suite 1425Arlington, VA
Program Officer: Sandra Chapman (sandra.chapman@navy.mil)
Period of performance: 2021 – 2022
Funding level: $98,100
Project goal: To enhance the CHMEP's ability to measure oxygen consumption and carbon monoxide production at rest and during exercise in mice and rats.
Specific aims: Equipment grant. 1) Acquire Oxymax Fast 2 Chamber System for mice and rats.

2) Acquire modular enclosed metabolic treadmill for mice 4 lanes with shock. 3) Acquire modular enclosed metabolic treadmill for rats 2 lanes with shock.
Project overlap: No overlap
Role: PI

Title: Characterizing hyperoxia-induced alterations in muscular physiology
Time commitment: 11.5% 1.38 calendar months
Supporting agency: Naval Experimental Diving Unit
321 Bullfinch Rd. Panama City, FL 32407
Administrative contact: Valerie Powell (valerie.powell@navy.mil)
Period of performance: 2021 – 2022
Funding level: $53,372 (Total costs)
Project goal: Identify potential causal factors of reduced human warfighter endurance performance during repeated dry dives while breathing 100% O_2 to develop future counter-measure strategies.
Specific aims: 1) Examine the the effects of hyperoxia exposures (HHE) on aerobic endurance performance. 2) Assess the influence of HHE on neuromuscular activity. 3) Evaluate the effects of HHE on systemic oxidative stress. 4) Examine the effect of HHE on muscle mitocondria. 5) Investigate the influence of HHE on skeletal muscle proteomics.
Project overlap: No overlap
Role: Associate investigator (PI Alexander Klemp)

Title: Enhanced assessment of oxidative tissue damage (N00014)
Time commitment: 0% 0 calendar months
Supporting agency: Office of Naval Research
One Liberty Center
875 N. Randolph Street, Suite 1425Arlington, VA
Program Officer: Sandra Chapman (sandra.chapman@navy.mil)
Period of performance: 2022 – 2023
Funding level: $92,589 (Total costs)
Project goal: To enhance the CHMEP's ability to assess oxidative tissue damage in current and future DOD supported research.
Specific aims: Equipment grant. 1) Acquire an enhanced chemiluminescent and fluorescent imaging system for detection of proteins and nucleic acids. 2) Acquire multi-unit respirometry system to measure isolated mitochondria and permeabilized muscle fiber oxygen consumption.
3) Acquire tissue dissociator to rapidly isolate mitochondria.
Project overlap: No overlap
Role: PI

PREVIOUS (within past 5 years):
Title: Effects of exercise on skeletal health in iron deficient female rats
Time commitment: 30% 3.6 calendar months
Supporting agency: Defense Health Program
US Army Medical Research and Development Command810 Schreider St.
Fort Detrick, MD
Agency's Contracting/Grants Officer: John Day (john.f.day26.ctr@mail.mil)
Period of performance: 2018 – 2020
Funding level: $252,000 (Total Costs)
Project goal: Determine if iron is essential for maintaining skeletal health during periods of loading.

Specific aims: 1) Determine if iron deficiency affects the oxygen carrying capacity of blood during 12-weeks of exercise training. 2) Determine how bone strength and function are impactedby iron deficiency during 12-weeks of exercise training.
Project overlap: No overlap
Role: PI 2018-2019, Co-investigator 2019-2020 (PI Jonathan Scott)

Title: Decrements in skeletal muscle performance and mitochondrial function following hypobaric exposure and enhancing oxyhemoglobin affinity to attenuate these effects in a rodentmodel
Time commitment: 10% 1.2 calendar months
Supporting agency: Office of Naval Research
One Liberty Center
875 N. Randolph Street, Suite 1425Arlington, VA
Period of performance: 2018 – 2020
Funding level: $318,988 (Total)
Project goal: Determine if 5-hydroxymethyl-2-furfural (5-HMF) is an effective countermeasureto hypoxemia.
Specific aims: 1) Determine whether increased oxyhemoglobin affinity can improve skeletal muscle strength and reduce time to fatigue following hypobaric hypoxic exposure. 2) Determine whether enhanced oxyhemoglobin affinity will stabilize aerobic exercise performance capacity during hypobaric hypoxia. 3) Explore the alterations in mitochondrial dynamics associated with high altitude, and whether improved skeletal muscle oxygenation improves mitochondrial function.
Project overlap: No overlap
Role: Co-investigator (PI Geoffrey Ciarlone)

Title: The effects of vitamin D on upper respiratory tract infections and immune function inMarine Corps recruits
Time commitment: 30% 3.6 calendar months
Supporting agency: Defense Health Program
US Army Medical Research and Development Command810 Schreider St.
Fort Detrick, MD
Agency's Contracting/Grants Officer: Karie Abernethy (karie.e.abernethy.ctr@mail.mil) Period of performance: 2015 – 2018
Funding level: $470,000 (Total)
Project goal: Determine if vitamin D supplementation decreases upper respiratory tract infections during Marine Corps basic training.
Specific aims: 1) Examine the relationship between serum vitamin D status and immune function. 2) Determine if vitamin D supplementation improves immune function.
Project overlap: No overlap
Role: PI

Title: Application of low dose inhaled carbon monoxide (CO) to attenuate diet-induced obesity
Time commitment: 40% 4.8 calendar months
Supporting agency: Uniformed Services University of the Health Sciences 4301 Jones Bridge Rd. Bethesda, MD 20814
Agency's Contracting/Grants Officer: Vice President for Research (301-295-3303) Period of performance: 2015 – 2018
Funding level: $90,000 (Total costs)
Project goal: Determine whether inhaled CO can prevent diet-induced obesity without affecting bone.

Specific aims: 1) Determine how CO influences skeletal muscle mitochondrial dynamics and function. 2) Determine the influence of CO with and without moderate exercise on bone strength and structure following a 10-week diet inducted obesity
Project overlap: No overlap
Role: PI

Overlap:
There is no scientific, budgetary, or effort overlap.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if
a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

Signature: Heath G. Gasier Digitally signed by Heath G. Gasier Date: 2021.08.24 10:31:46 -04'00'

Date: 08/24/2021

SPOC – 08/23/21 – [redacted] (Anesthesiology)
Reviewed by: [redacted] (Anesthesiology) – 08/24/21

Abstract

Hyperbaric oxygen (HBO_2) increases the partial pressure of oxygen (PO_2) in the lungs, blood and tissues. The pulmonary and central nervous systems are very sensitive to changes in PO_2, and high levels can cause oxidative tissue damage that can be lethal. The USN established single-depth oxygen exposure limits that serve to reduce the probability of developing central nervous system oxygen toxicity (CNS-OT) that may culminate in tonic-clonic seizures, thus are restrictive for depth and time. For this reason, research has been directed towards understanding the mechanisms of CNS-OT with the goal of finding targeted prevention strategies. It is known that increasing the GABA-to-glutamate ratio delays seizure latency, and this can be accomplished by administering FDA approved antiepileptic drugs (AEDs). We have determined in mice that a single 60-minute exposure to HBO_2 at 5 ATA causes DNA oxidation, inflammation and selective degradation of mitochondria, i.e., mitophagy, mainly in the cerebellum and hippocampus. This is followed by mitochondrial biogenesis and injury resolution within 72-hours. The AED tiagabine (TGB) attenuated acute brain injury and enhanced mitochondrial biogenesis. Because exposure to HBO_2 increases sensitivity to CNS-OT, which is linked to oxidative lung injury, the *main objective* in this application is to determine how HBO_2 and GABA influence repeated exposure-sensitivity to oxidative tissue injury. A *secondary objective* is to determine the impact of elevated GABA levels on running performance after HBO_2. Our *central hypothesis* is that increasing the GABA-to-glutamate ratio will shorten the time course of oxidative injury repair, decreasing repeated HBO_2 exposure sensitivity. The *rationale* is that it will determine if maintaining GABA levels affords protection against oxidative tissue damage with repeated exposure to HBO_2 and offer a strong scientific framework for testing GABAergic AEDs in humans. To test our hypothesis and attain our objective, the following three *specific aims* will be pursued: *1)* Determine in C57BL/6 mice if GABA influences repeated HBO_2 exposure sensitivity to seizures and lung injury. *2)* Determine in *mito*-QC mice the impact of HBO_2 and GABA on recovery from oxidative tissue damage. *3)* Determine in C57BL/6 mice if increased GABA impairs running performance after HBO_2. To accomplish this research, we will use wild type and transgenic mice with fluorescent labels for mitochondrial architecture and mitophagy (*mito*-QC) and expose them to single or repeated HBO_2 exposures at 5 ATA. Running performance will be tested after HBO_2 at 3 ATA. GABA metabolism and oxidative injury in the brain and lung will be measured by ultra-performance liquid chromatography-tandem mass spectrometry, immunohistochemistry and immunofluorescence, immunoblotting and assays. The proposed research is *significant* because it will lay the groundwork for testing GABAergic AEDs in humans performing work in HBO_2. The proximate *expected outcomes* of this work is knowledge of how GABA influences the time course of oxidative injury repair in the brains and lungs of mice exposed to repeated HBO_2. The results will have an important *positive impact* immediately because they will establish new function(s) of GABA, and potentially lead to increased oxygen exposure limits. The proposed research is *relevant to warfighters* because breathing 100% oxygen during diving operations can impair work performance and mitigation approaches are needed. This proposal addresses two of Undersea Medicine & Performance Concentration Areas: mitigation of hyperbaric oxygen toxicity and diver performance optimization.

"*Approved for Public Release*"

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V. Facilities & Equipment

The CHMEP is among the largest laboratories of its kind in the world. Within the CHMEP, the FG Hall Environmental Laboratory, established in 1963, contains a 432 ft^2 chamber complex (eight chambers) capable of compression to 3,600 ft. of sea water or decompression to 100,000 ft. of altitude. There are also several hyper- and hypobaric chambers devoted to animal research. Adjoined to the FG Hall Environmental Laboratory are rooms dedicated to human and animal physiological testing, tissue handling and processing, cell culture, molecular biology, protein biochemistry, animal surgery, gas analysis and light/fluorescent microscopy. A biomedical electronics and machine shop provide in-house design and construction capability. The CHMEP is housed within Duke Medical Center, with resuscitation equipment and facilities for administering oxygen and intravenous drugs. There is immediate access to critical care units, hospital labs, radiology and MR imaging. Material and procedural adequacy of the chamber facility have been certified by the US Navy. All studies proposed in this application will be performed at the CHMEP.

Laboratory equipment includes basic bench instruments such as 3 tissue homogenizers and 2 sonicators, 2 analytical balances, 3 water baths, 2 pH/ion-analyzers, 2 low-temperature (-80 °C) and 4 -20 °C freezers, a Hitachi U2000 spectrophotometer, and a Shimadzu UV-visible scanning spectrophotometer. Other equipment includes a Prizm spectrofluorometer, a Tecan Safire temperature controlled multi-mode plate reader, a multi-channel differential spectrophotometer with low temperature capability, a dual oxygraph with two-channel recorder, Reichert dissecting microscopes, a Sorvall RC-5 Superspeed Centrifuge, a Beckman Ultra 80 ultracentrifuge with titanium rotor, 3 bench-top centrifuges (one with refrigeration), and 3 complete HPLC systems with electrochemical, fluorescence and optical detection capabilities. In addition, the laboratory contains multiple Hoefer and Biorad PAGE systems with power supplies, and an electrophoresis DNA and RNA gel system. The Duke laboratory contains a BSL2 hood, two Nikon Eclipse high resolution fluorescent microscopes equipped with color and monochromic cameras and NIS-Elements software, a Leitz Vario-Orthomat microscope with camera, a darkroom Philips X-ray film developer, and a BioSpectrum Imaging System. For molecular studies, TaqMan-real time PCR, ABI Stepone Plus and 7500 Sequence detectors are available. For conventional PCR, 4 Veriti Thermal Cyclers, a hybridization oven, and a 10 Owl horizontal gel separation system are available. Also available is an image analysis scanner and software (BioRad) and a Tecan GENios multimode microplate reader.

Dr. Gasier has received an ONR DURIP (FOA NOOO14-20-2-F004) in 2021 to procure a metabolic testing system with metabolic treadmill for mice and rats that will be used for Aim 3 in this proposed research.

Cover Page

BAA Number: N00014-21-S-B001

Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO_2 Exposure

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Period of Performance: 1/1/2022 – 12/31/2024

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I. TECHNICAL APPROACH

Significance

Hyperbaric oxygen (HBO_2) increases the partial pressure of oxygen (PO_2) in the lungs, blood and tissues. The pulmonary and central nervous systems are very sensitive to changes in PO_2, and high levels can cause oxidative tissue damage that can be lethal. The USN established single-depth oxygen exposure limits from a body of research conducted at the Naval Experimental Diving Unit, reported in 1986 (1). The exposure limits serve to reduce the probability of developing central nervous system oxygen toxicity (CNS-OT) that may culminate in tonic-clonic seizures, thus are restrictive for depth and time. This dive schedule constrains military diving capabilities, and may need to be exceeded during operations placing divers at increased risk. For this reason, research has been directed towards understanding the mechanisms of CNS-OT with the goal of finding targeted prevention strategies.

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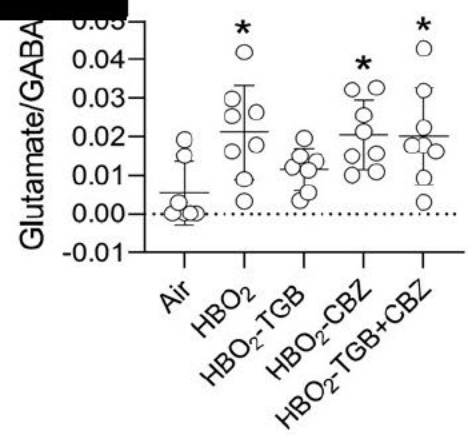

Fig. 1. Forebrain excitotoxicity index in C57BL/6 mice exposed to HBO_2 at 5 ATA for 30 min. Mice received an IP injection of 0.9% NaCl, TGB (4.8 mg/kg), CBZ (16.4 mg/kg), or TGB+CBZ 30 min before HBO_2. Brains were harvested post-HBO_2 for measurement of glutamate and GABA using immunoassays. $*P < 0.05$ *vs.* air control (one-way ANOVA). Values are means ± SD, n = 7-8 mice/group. Unpublished data.

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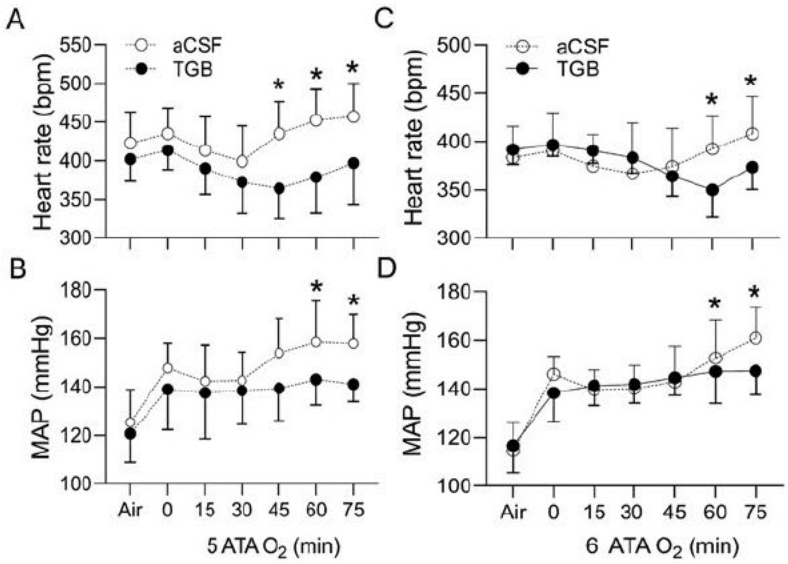

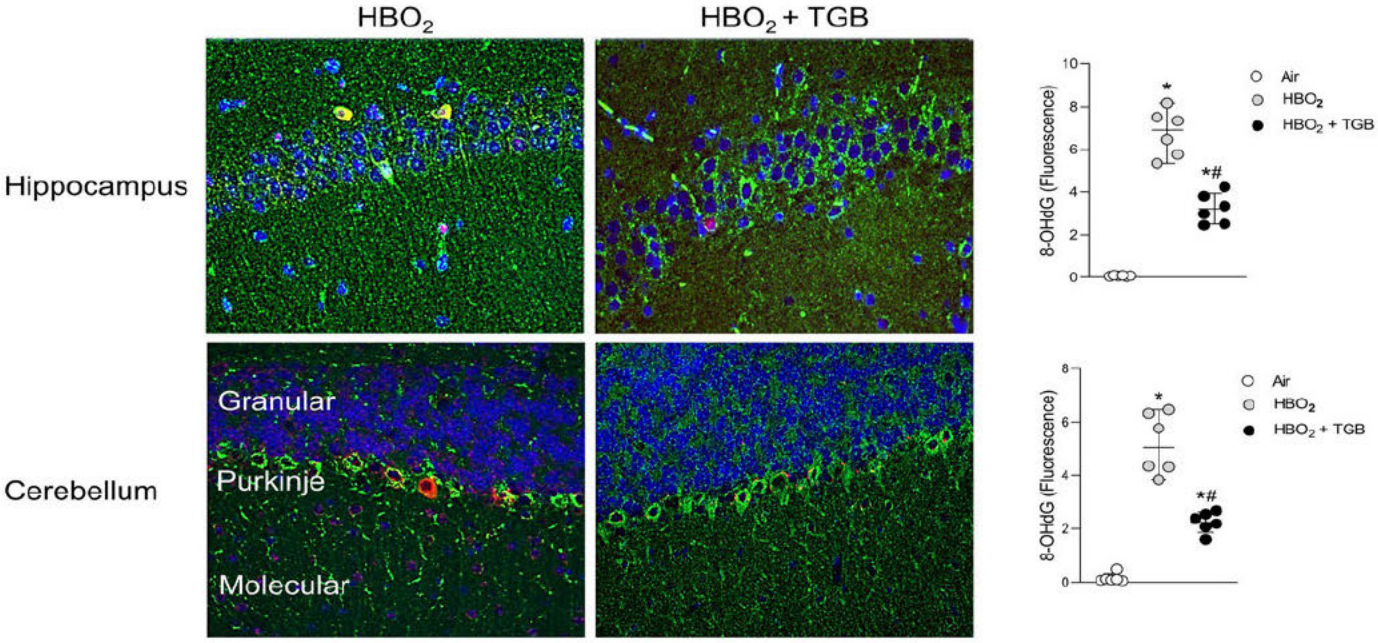

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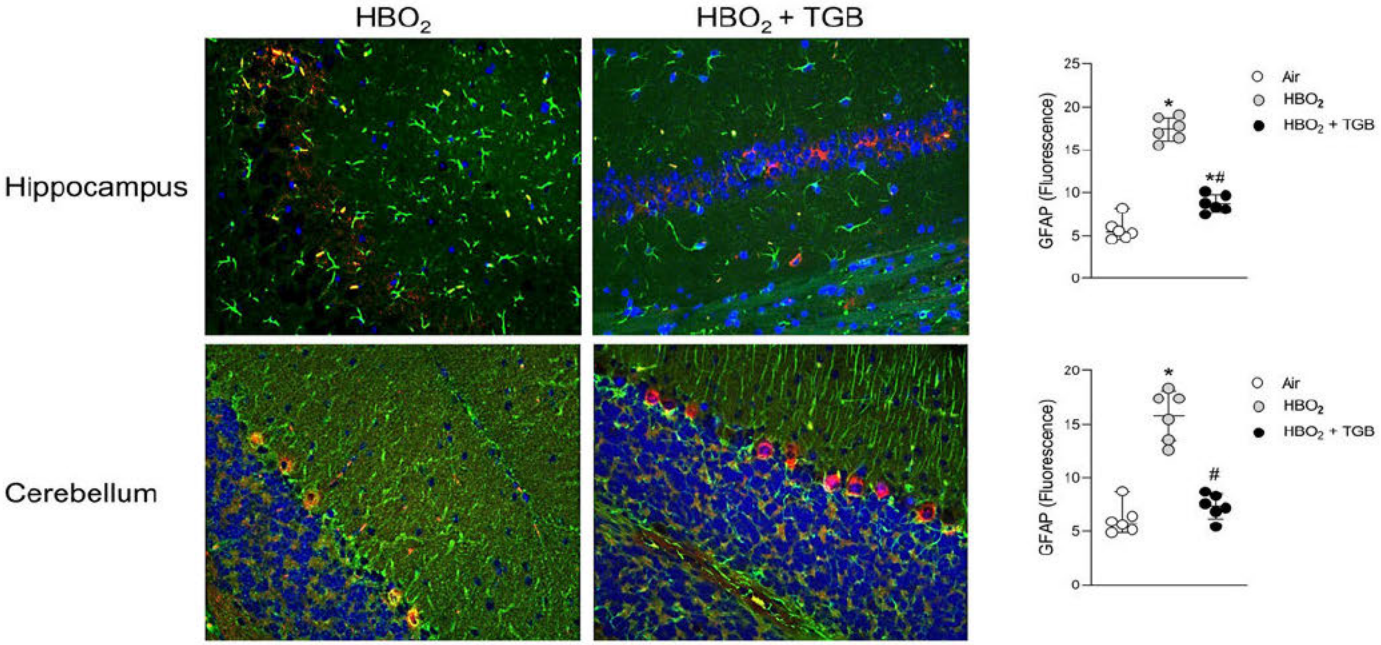

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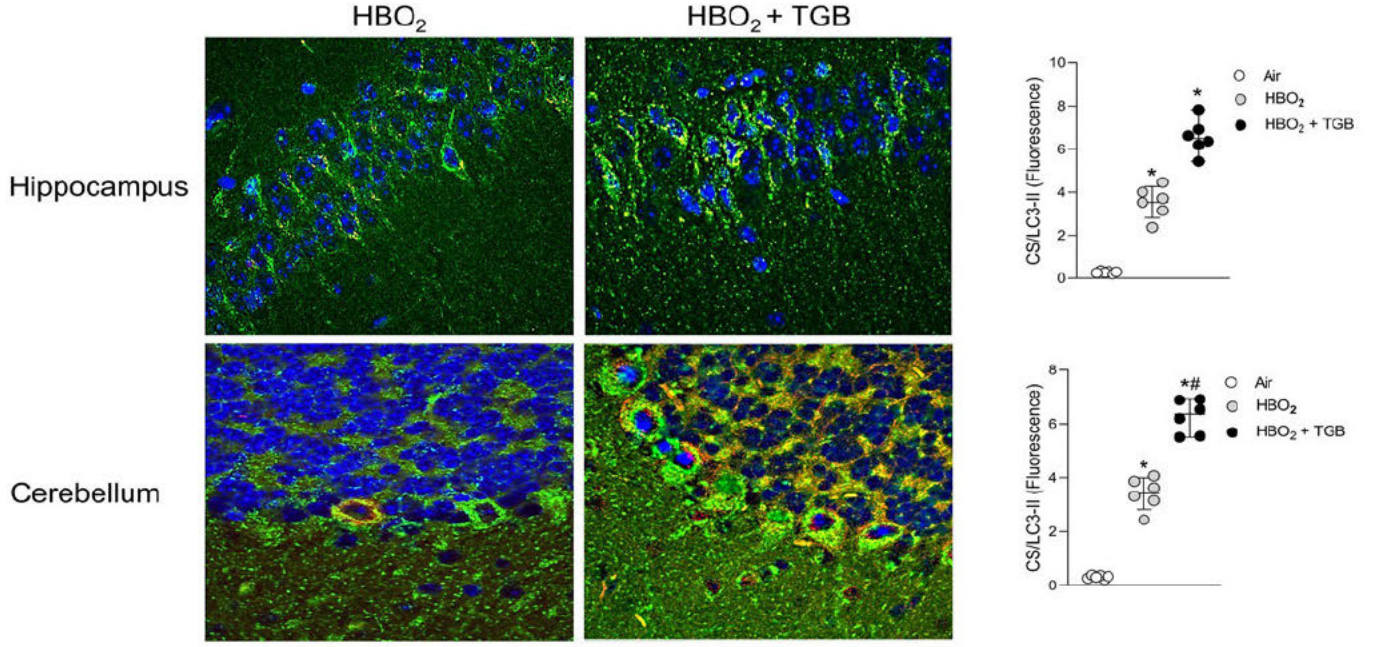

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Innovation

The status quo as it pertains to studying CNS- and pulmonary-OT is to expose cells, animals or humans to a single HBO_2 exposure. This approach has advanced our understanding of the pathophysiology of these linked complications, and led to testing targeted mitigation strategies. Less information is available on why exposure to repeated HBO_2 increases sensitivity to CNS-OT and lung injury (10-13). Chavko et al. (11) and Liu et al. (13) reported an increase in nitric oxide synthase and NO content in the brains after repeated HBO_2 exposures. Because oxidant production is increased in HBO_2, this work did not identify a cause, but rather an effect of repeated HBO_2 exposures. (b) (4)

(b) (4) [redacted] This innovative approach is expected to lead to novel findings that will open new horizons for improved targeted treatments, and lead to testing GABAergic AEDs in human studies.

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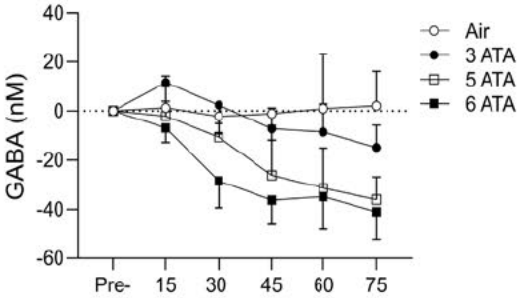

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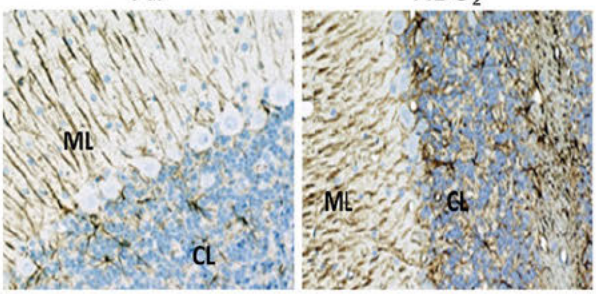

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$\dot{V}O_{2max}$ testing, mice will be humanely euthanized. Lungs will be excised, weighed and dried over 72-hours, and weighed again. The wet-to-dry ratio will provide indication of oxidative lung injury (36). Blood will be collected for measurement of glucose and lactate using an in-house blood gas analyzer.

Exercise Testing. Habituation to the treadmill will occur over four 10-minute sessions. The grade will be set at 0%. Velocity will be increased from 0 to 3, 6 and 9 m/min. Sessions will be interspersed by 24 hours. After habituation, mice will complete a $\dot{V}O_{2max}$ test in an enclosed metabolic chamber (Columbus Instruments). Mice will complete the test at a 0% grade and starting speed of 3 m/minute. Velocity will be increased by 0.05 m/second until exhaustion, which should be 15 ± 1 minutes (52). $\dot{V}O_2$ and $\dot{V}CO_2$ will be measured every 3 seconds. RER will be calculated from the $\dot{V}CO_2$-to-$\dot{V}O_2$ ratio. $\dot{V}O_{2max}$ will be considered the highest $\dot{V}O_2$ averaged over 15 seconds. Blood lactate and RER will be used to confirm $\dot{V}O_{2max}$, i.e. lactate > 8 mM and RER > 1. If these criteria are not met, we will consider the highest VO_2 attained the $\dot{V}O_{2peak}$.

Statistics. In order to determine the effect of condition and treatment on running performance, data will be analyzed using a two-way ANOVA.

Expected Outcomes. The outcome of Aim 3 will be an understanding of GABA and HBO_2 on running performance. This knowledge will be useful for transitioning into human studies.

Potential Problems & Alternatives. Not all rodents learn to run at the same pace and this is often challenging. For this reason, additional habituation sessions may be required. The PI is experienced with rodent exercise training (53-55) and will oversee all habituation and $\dot{V}O_{2max}$ testing. We do not anticipate any premature death with this experimental protocol.

Naval Relevance

In 1957, USN CAPT George Bond F. Bond, the "Father of Saturation Diving," submitted a proposal to the Bureau of Medicine and Surgery to answer two questions: "How long can a man stay down? How deep can a man go?" (56). The goal of the NAVSEA Diving Program (2011-2025) is still to extend the amount of time a diver can perform operations in the water (57). For over 60 years, the goal has remained unchanged. This research will address two exposure-limiting problems and two of ONR Undersea Medicine & Performance Research Concentration Areas: mitigation of HBO_2 toxicity and diver performance optimization. This work will parallel Dr. David Eckmann's (Ohio State University) research that is focused on understanding the effects of oxygen on the mechanisms of cellular injury in the lung using in vitro models. This research will also complement Dr. Jay Dean's (University of South Florida) research that is focused on testing mitochondrial antioxidant therapies in extreme HBO_2. By applying different approaches to study how oxygen becomes toxic to cells and tissues, knowledge gaps will be filled quicker. Safe and efficacious mitigation strategies for oxygen toxicity will enable divers to descend to greater depths for longer periods of time, and decompress more rapidly since oxygen prevents and is used to treat decompression illness. The knowledge from this research will also be important for submarine survival and escape in the event the hull integrity becomes compromised. Maintaining Undersea Superiority is critical for preserving an elite and battle ready USN Force. This research directly supports this mission.

Project Schedule and Milestones

The study timeline is shown in **Table 1**. This study requires IACUC approval. ONR Award No. N00014-18-1-2702 has been transferred to Dr. Gasier, the PI on the IACUC approved protocol (A023-19-01). This protocol will be amended as a major modification to include animal use for the proposed research. Under project N00014-18-1-2702 and in year 1, (b) (4) [redacted]

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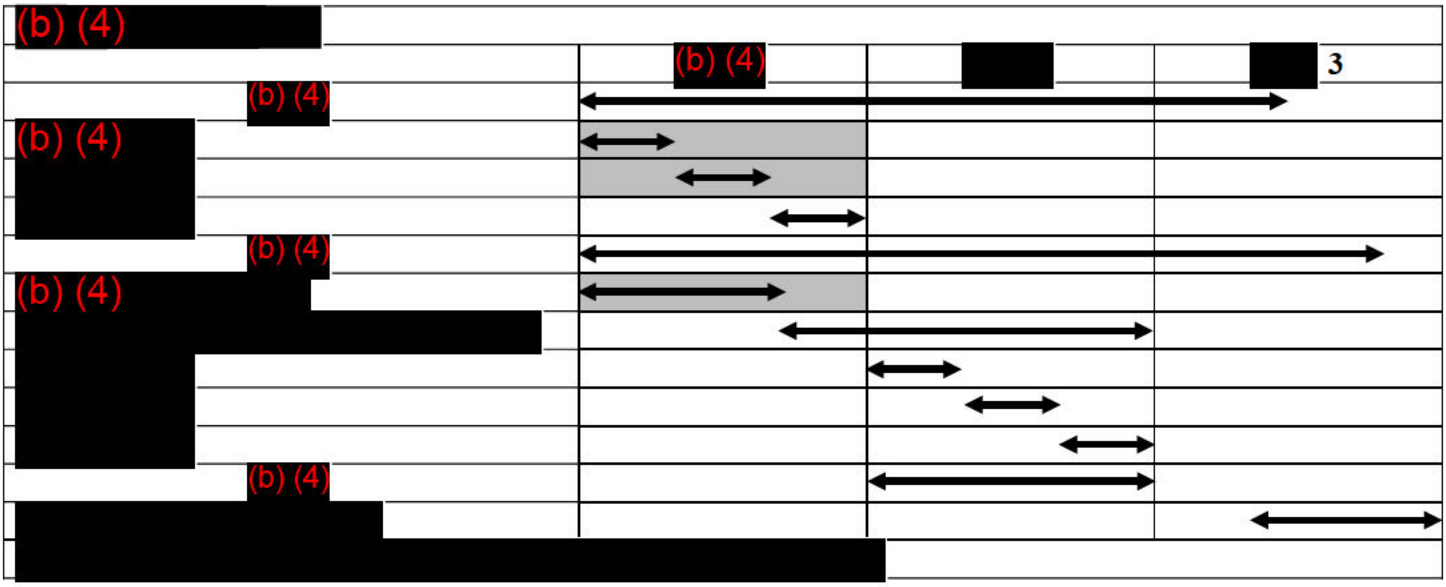
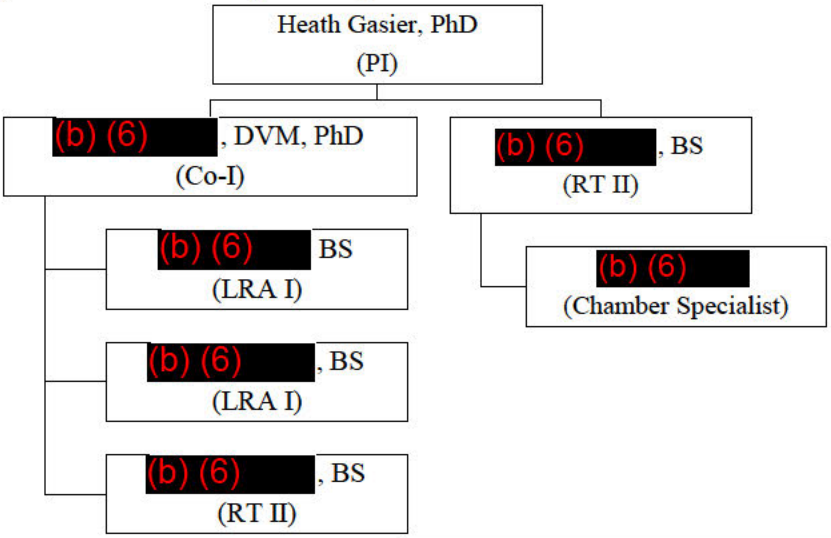


II. Management Approach

The proposed research will be performed at the Duke CHMEP. The study PI, Heath Gasier, will assume overall responsibility of the project and lead the research team consisting of Dr. (b) (6) (Co-I), (b) (6) Lab Research Analyst I, LRA), (b) (6) (LRA I), (b) (6) (RT II), (b) (6) (RT II) and (b) (6) Hyperbaric Chamber Specialist).



Dr. (b) (6) will be responsible for directing (b) (6) I. Dr. (b) (6) will perform the bulk of the fluorescence and confocal imaging, analyze data, and assist with preparing reports and writing manuscripts. (b) (6) will be responsible for monitoring and breeding the mito-QC mice once they are transferred to the Division of Lab Animal Resources. (b) (6) will be responsible for purchasing, data management, tissue and blood collection, immunofluorescence, immunohistochemistry and immunoblotting. (b) (6) will be responsible for monitoring the animals during HBO_2 exposures and

recording seizure latencies, exercise training, immunofluorescence, immunohistochemistry and immunoblotting, and ELISAs. (b) (6) will be responsible for directing hyperbaric chamber operations. (b) (6) will be responsible for performing the HBO_2 exposures.

Research meetings are held every Monday morning to plan chamber experiments and assess personnel requirements. Each of the research team members have offices within the CHMEP and communicate on a daily basis. (b) (6) uploads data into Duke University's supported LabArchives eResearch Notebook. Data storage is secure and uses a cloud-based server. This allows access to approved site users and reduces data transmission time.

III. PI Qualifications

The PI will be Heath G. Gasier. I joined the CHMEP (Department of Anesthesiology) in August of 2019 after retiring from the USN as a Research Physiologist. I have served as a PI on military related research since 2006, and have received funding from the ONR, DHP and DMRDP. From 2013-2105, I completed the first out-of-service fellowship in hyperbaric physiology at the CHMEP under the direction of (b) (6), a pioneer in the field who has been funded by ONR for 30+ years. My research is focused on understanding how oxidative damage to the brain, lung and skeletal muscle alters function. The work that I have been a part of has led to testing the efficacy of adrenoceptor antagonists and AEDs in extreme HBO_2. From this body of work, we have learned that the non-selective β-receptor antagonist propranolol and several AEDs, e.g. TGB, lamotrigine, carbamazepine and gabapentin, are efficacious in delaying HBO_2 seizures. This proposal is an extension of this work and important for transitioning into human testing. My colleague, Dr. (b) (6), will be a Co-I. She is an (b) (6) in the Department of Anesthesiology and has been a part of the CHEMP for 21 years. She is an expert in pathology and the transcriptional regulation of mitochondrial dynamics. Our knowledge and skills complement each other and will result in the successful completion of this research.

IV. Responsibility

All HBO_2 exposures will be conducted in chamber Golf, the CHMEP's dedicated animal chamber (390.5 $ft.^3$). Exercise habituation and testing will be completed in an adjacent IACUC authorized rodent housing room that is temperature controlled and on a 12 h light and dark cycle. Euthanasia and tissue collection will be performed in adjacent animal surgical laboratories. Assays, immunofluorescence, immunohistochemistry and immunoblotting will be completed in adjacent biochemistry and imaging laboratories. The *mito*-QC mice will be generated by the Duke Rodent Genetic Engineering Services. Brain and lung tissue will be sent to the Duke Proteomics and Metabolomics Core Facility for measurement of GABA, glutamine and glutamate.

The majority of my time (95%) is dedicated to research, which includes directing laboratory technicians and mentoring students. The remainder of my effort is devoted to teaching, committees and peer review. I have sufficient time to execute this research.

I successfully managed federal awards since 2010 and always meet project milestones. I am diligent with timelines, budget management, and ensuring funds are expended/allocated in accordance with the proposal. The ONR awards N00014-21-1-2279 and N00014-18-1-2702 will be completed in April of 2022. The NAVSEA award N00024-21-C-4310 will be completed in March of 2022.

I served in the military for 27 years, both enlisted and officer, and believe this has contributed to my honesty and practice of strong moral and ethical principles. From 2010-2013, I was the Research Integrity Leader and Vice Chair of Scientific Review Board at the Naval Submarine Medical Research Laboratory, Groton, CT, highlighting my commitment to responsible conduct in research. In addition, I complete annual research ethics training.

My current position is Assistant Professor (non-tenured faculty) in the Department of Anesthesiology and meet the qualifications to serve as a PI on federal grants.

I am detail-oriented and enjoy organizing and planning experiments, leading teams and trouble shooting. High quality presentations, reports and peer reviewed publications is testament of my knowledge, skills and

abilities. I always master or familiarize myself with techniques that are appropriate to meet study objectives, and form collaborations with others who are more knowledgeable. As a PI, I ensure that the research staff works in a safe and friendly environment, and that quality standards are adhered to and documented where need be. My time spent as a military officer and PI has provided me with the necessary skills to maintain a structured and effective research laboratory that will ensure the success of this research.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Animal Use Protocol

Protocol History || Animals Used to Date || Animal Welfare Inspection Report

Protocol Number: S19154

Meeting Date: 11/13/2019

Submitted By: (b) (6)
Email: (b) (6)(b) (6)
Work Phone:
Emergency Phone: (b) (6)

P.I.Name: (b) (6)(b) (6)
E-Mail: (b) (6)(b) (6)
Work Phone:

University Title: (b) (6)
Department: (b) (6)
Emergency Phone: (b) (6)

. General Information *Questions 1-5*

1. Type of Protocol

Research

2. Protocol Title

Nitrogen gas uptake and excretion during compression/decompression to simulate diving

3. Animal Species

Rat - Rattus norvegicus

4. Lay Overview

State the research (or teaching) goals in two or three sentences, using language that can be understood by a lay person. Please avoid technical terms and acronyms.

When diving and breathing gas under water the body takes up additional nitrogen gas. This is due to the higher environmental pressure caused by the water compressing the gas in the lungs. When coming back to the surface (decompression) this gas will be excreted. If the amount of gas is too high there will be problems with decompression sickness (a medical diagnosis) due to creation of gas bubbles in various tissues in the body (similar to shaking a soda bottle that create carbon dioxide bubbles). The medical problems depend on the amount of gas and where the bubbles appear. A bubble in the skin may cause an itch, while a bubble in the brain may cause a stroke or death. This is a problem in recreational, commercial, and military operations. This has been studied for over 100 years and still only empirical methods to dive reasonably safe exist. The exact pathophysiology is not known. Current methods can only detect bubbles in venous blood with ultrasound. No method so far has shown tissue bubbles or gas after compression/decompression. The aim of this project is to develop a new method using radioactive gas to study how the nitrogen gas in air affects the body during compression and decompression. We will administer the gas in a compression chamber that simulates diving (but dry, in a lab). We will study the pathophysiology with compression/decompression with animal experiments and due to the morbidity of such experiments we will use anesthetized animals that will be euthanized after the experiments and before waking up to avoid suffering. The possibility to do this now is due to recent developments in imaging technology that can scan and detect radioactive gases.

Describe how this research (or teaching) will benefit society, advance knowledge, or benefit human or animal health.

Operational procedures are still being developed and decompression sickness is still a major problem and mystery despite 100 years of ongoing research efforts. Our new proposed method aims to develop basic knowledge into the mechanisms and possibly solve this problem so that diving can be done more safely in recreational, commercial and military operations. The extension of this research is to improve human health and safety.

Briefly summarize your research in 2 or 3 sentences. Details will be described below in Question 12.

We will have rats breathing radioactive gas while compressed in a pressure chamber to simulate diving. After that they will be imaged with a PET-camera to measure where in the body the gas was distributed and how it is released after compression/decompression ended. All techniques have been done previously in animals and humans (i.e diving/pressurization, breathing gas, PET-imaging) but our new combination has never been explored. The combination will yield new interesting data.

5. Study Endpoint for Animals

- [ ] Acute/Terminal (animal never awakens from initial procedure)
- [x] *Survival (how long) for*

24 hrs

I. Personnel

Existing Personnel

[redacted]
Alternate Contact

☐ Principal Investigator ☑ Alternate Contact - Edit Access ☐ No Edit Access

☐ No Animal Contact	Surgery-Survival-Major	Surgery-Survival-Minor	☐ Surgery-Terminal
☐ Injections	Post-op Surgical Care	Surgical Records	☑ Anesthesia
☑ Euthanasia	Breeding Mgmt.	Drug-Diet-Admin	☑ Hazard Administration
☑ Blood/Tissue Collect	☑ Handling/Husbandry	☑ Observation	☐ Noninvasive Testing

[redacted]
Principal Investigator

☑ Principal Investigator ☐ Alternate Contact - Edit Access ☐ No Edit Access

☐ No Animal Contact	Surgery-Survival-Major	Surgery-Survival-Minor	☐ Surgery-Terminal
☐ Injections	Post-op Surgical Care	Surgical Records	☑ Anesthesia
☑ Euthanasia	Breeding Mgmt.	Drug-Diet-Admin	☑ Hazard Administration
☑ Blood/Tissue Collect	☑ Handling/Husbandry	☑ Observation	☐ Noninvasive Testing

[redacted]
Alternate Contact

☐ Principal Investigator ☑ Alternate Contact - Edit Access ☐ No Edit Access

☑ No Animal Contact	Surgery-Survival-Major	Surgery-Survival-Minor	Surgery-Terminal
Injections	Post-op Surgical Care	Surgical Records	Anesthesia
Euthanasia	Breeding Mgmt.	Drug-Diet-Admin	Hazard Administration
☐ Blood/Tissue Collect	☐ Handling/Husbandry	☐ Observation	☐ Noninvasive Testing

II. Federally Required Information and Assurances *Questions 6-9*

In accordance with Public Law 89-544 (Animal Welfare Act of August 24, 1966), Public Law 91-579 (Animal Welfare Act Amendments of 1970), Public Law 94-279 (Animal Welfare Act Amendments of 1976), Public Law 99-198 (Food Security Act of 1985, Subtitle F - Animal Welfare), Code of Federal Regulations, Title 9, Chapter 1, Subchapter A - Animal Welfare, and the Public Health Service Policy on Humane Care and Use of Animals, the UCSD Institutional Animal Care and Use Committee is required to obtain the following:

6. Why are living animals required for your study?
Why can't you use replacements such as cell culture, computer modeling or other non-animal models? Check all that apply:

- ☑ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems
- ☑ There is not enough information known about the processes being studied to design nonliving models.
- ☐ Preclinical studies in living animals are required by federal regulations prior to human testing.
- ☐ This is a behavioral, learning or developmental study.

Other

7. Why is the proposed species the most appropriate?
Why can't a less sentient, phylogenetically lower species be used?
Check all that apply:

- ☐ A large database exists for this species allowing comparisons with previous data.
- ☐ The anatomy, genetics, physiology or behavior of this species is uniquely suited to the study proposed. Describe below.
- ☐ This is the phylogenetically lowest species that provides adequate size, tissue or anatomy for the proposed study.
- ☐ This species provides a particularly good model for duplicating the human situation. Describe below.
- ☐ The results will be directly applicable to the health or care of this species. Describe below.
- ☑ Previous studies using this species formed the background of this project.
- ☐ The species has unique features that make it the best choice available for this study. Describe below.

Explanation

Prior animal studies in decompression sickness have primarily used rats and sheep. Using rats for these experiments (instead of alternatives as mice, guinea pigs etc) enables us to copy methods and protocols known to create the desired effect: we need to produce a limited decompression stress due to nitrogen gas uptake and bubble formation. Also, rats have been shown to function well as experimental animals in compression-decompression, i.e they can handle the pressure changes similar to a human subject diving.

8. Duplication of Research

☑ By checking here I certify that in planning this experiment I have reviewed the relevant literature (by computer database literature search, use of comprehensive review articles, consultation with Animal Welfare Information Center, etc). Based on the literature, I certify that the activities involving animals described in this protocol do not unnecessarily duplicate previous research. I assure that I will retain my search records for three years past the end of the animal studies and that these search records will be available to inspectors at any time.

9. Consideration of Alternatives to Painful Procedures
Are you:

☐ Using a warm-blooded vertebrate species OTHER THAN rats, mice or birds.

☑ Proposing procedures that fall into the USDA Pain Category D or E (causing more than momentary or slight pain or distress).

V. Animal Requirements for the 3-Year Approval Period *Questions 11-13*

11. Strains/Breeds

Breed (Strain/Genotype)	Genetic Alteration	Phenotype/Health Issues	Age (adult, juv, fetus, etc.)
Sprague Dawley rats	None	N/A	adult

12. Research Plan for the 3-Year Approval Period
Please read the instructions carefully. **This is the most important section of the protocol**. This is the section where you 1) provide scientific justification for the requested number of animals to be used in the three year period (before the next rewrite is due) and 2) describe the studies to be performed including, as appropriate, experimental and control groups, manipulations or procedures performed, time courses, measurements/experimental end points and final disposition of the animal subjects. The reviewers must be able to understand the experimental rationale, research plan, and comprehensive history of the animals. One text box should be used for each study or set of closely related studies. Generally, any animal to be used should be assigned to only one text box. Get detailed help and examples. If you will not be breeding animals, click on the REMOVE button for the group '"Breeding Colony Maintenance."

Number of Animals in Each Category of Pain/Distress

Study or Experiment Name	C	D	E
1. Exercise and Ultrasound of Venous Gas Embolism Detection	0	25	0

Briefly describe the scientific question or rationale to be addressed or, if not performing an experiment, the purpose served by use of animal subjects. Describe the study to be performed. This generally involves describing the experimental design including all experimental groups and controls, sample sizes, procedures and/or manipulations, timelines, and endpoints. If a procedure, surgery, or collection is performed multiple times, this must be described here. For each text box, provide a calculation of the total number of animals requested and identify the appropriate pain/distress category for the animals. Do not include procedural details here; procedures should be described in the sections below. Likewise describe in Question 13 how sample sizes are determined. A reviewer should be able to understand the complete time-course of procedures on each animal that you describe in the studies below.

We will create a situation with differences in amount of blood gas bubbles vs gas in solution in a live animal. This can be performed by recreating a rat experiment published in 2001 where it was shown that that exercise the day before compression/decompression reduce the amount of bubbles to the extent that mortality rates changed significantly. Bubbles were studied by ultrasound then as we will do now as well. The published compression/decompression created vast amounts of bubbles in the control animals after a 45 minute compression. We do need to investigate various amounts of bubbles to study the effect of various levels of gas in tissues. Therefore we will test a slightly less strenuous protocol as well, comparing 30 minutes of compression with 45 minutes.
We will have to test and recreate this including calibration of exercise load, and confirm that this procedure works in the currently available strain of rats etc.
Rats will be split into four equal groups:
control (no exercise) and 30 minutes compression
control (no exercise) and 45 minutes compression
exercise and 30 minutes compression
exercise and 45 minutes compression

Exercise groups:
The exercise cohort will be placed onto a motorized, rat sized treadmill for 1.5 hours of exercise 18-20 hrs prior to compression in the chamber. The treadmill will force them to run according to set parameters (the speed of the treadmill run is published data and is noted below in the Other Procedures box. We will most likely use a commercially available treadmill with an electric grid at the rear of the treadmill to provide shocks if rats do not run willingly. Shocks will be no greater than 2mA. We will also consider systems with the option to use air puffs as an alternative encouragement mechanism. All rats will be monitored during exercise to reduce intensity or finish the exercise bout if rats are unable to run (to avoid multiple shocks). This exercise protocol follows previously published data, however if we find that the rats we use are unable to reliably run for the established 1.5hr period, we will reduce the time, intensity, or both. After this exercise they will go back to their regular cage until the next day.

Compression/Decompression, all animals.
Animals will first be anesthetized (isoflurane will be added to the mixture at 3-5% of the total). They will be shaved and naired over the heart. They will be placed in the chamber for compression/decompression receiving normal pressurized air. Compression/decompression using the same profile as Wisløff and Brubakk 2001 (again, described in the Other Procedures box) with chamber compression to 700 kPa, for 30 or 45 minutes.
Following the compression cycle, rats will be taken out of the chamber and loosely restrained on a warmed imaging stage, while still anesthetized. Warmed ultrasound gel will be used to couple the transducer to the animal over the chest, and imaging will occur over the course of 1 to 2 hours. Imaging modes may include normal B-mode, color Doppler mode, or contrast mode. All modes utilize similar energy levels, and all are well below the threshold for mechanical or thermal tissue disruption. Total anesthetization time will be 2-3 hrs. Upon completion of the scan, each rat will be euthanized (while still under anesthesia). Decompression can cause long term damage and pain, thus rats will not be allowed to wake up.

The 45 minute compression/decompression profile has, according to the publication, a 50% rate of mortality in the non-exercised rats and significant differences in bubble load compared to the exercised group. It is believed the bubbles contribute to mortality. We expect this part to verify the old experiments and tune our procedures so we have the right size of rat, the correct exercise intensity, and thus a reproducible method to change the amount of gas bubbles during decompression after compression in a chamber. We hypothesize that the 30 minute compression to yield less bubbles and less mortality but still significantly large differences between control/exercise that we can use that as an experimental protocol. Please note that while animals may die from decompression sickness, no animal will ever be conscious after compression has begun, until euthanasia. Any animals that die will be heavily anesthetized, as will all surviving animals until they are euthanized. Since death from decompression sickness can be quick and sudden we are unlikely to be able to consistently intervene and euthanize dying animals before the sickness kills them. Further, the bubbles that lead to death are exactly what we plan to image, so seeing how they develop in dying and surviving animals at very late stages is important.
Due to the individual variation in sensitivity to decompression and bubble formation we anticipate a need to do 5 animals in each of the four experimental groups with an additional reserve of 5 animals if some experiments would have to be repeated or failed due to logistical or technical challenges.

Study or Experiment Name	C	D	E
2. Breathing of radioactive gas in the PET at normal atmospheric pressure.	0	6	0

Briefly describe the scientific question or rationale to be addressed or, if not performing an experiment, the purpose served by use of animal subjects. Describe the study to be performed. This generally involves describing the experimental design including all experimental groups and controls, sample sizes, procedures and/or manipulations, timelines, and endpoints. If a procedure, surgery, or collection is performed multiple times, this must be described here. For each text box, provide a calculation of the total number of animals requested and identify the appropriate pain/distress category for the animals. Do not include procedural details here; procedures should be described in the sections below. Likewise describe in Question 13 how sample sizes are determined. A reviewer should be able to understand the complete time-course of procedures on each animal that you describe in the studies below.

We will set up and test the technique of breathing or rebreathing radioactive gas (13N2) on a few rats in normobaric conditions, i.e no compression, no chamber.

Each rat will be anesthetized (with isoflurane) and the gas line will be securely attached to their face via a sealed nosecone and rebreather device. The rat will then be placed upon the micro-PET (Vista DR from GE Healthcare) gantry bed. The animals will then breathe pure oxygen for 1 hour to off-gas nitrogen (i.e reduce the naturally occurring non-radioactive nitrogen in their body tissues and gas in the lungs. They will be advanced into the imager until their heads rest in the center of the field of view. Rats will be breathing off a rebreather system capable of switching gases for recycling the inert gas while scrubbing carbon dioxide, as well as switching to a separate collection of exhaled gas:

The radioactive 13N2 will be mixed with oxygen to generate "air" with 21% oxygen in a "bag" (lead shielded). From that bag will the rat breathe via a tube that connects to the nose-cone. Isoflurane will be added into this tube. When the rat exhales the gas can go two ways (the experimenter can switch a valve), either will the exhaled gas go to a collection bag were it will be allowed to decay all radioactivity (a few hours) before disposed according to the procedures to handle the isoflurane content. The other option is that the exhaled gas goes back into the rebreatherbag via a scrubber system that absorbs carbon dioxide (standard technology for a respirator system). This version may need a small fan to blow the gas through the scrubber material before going back to the "breathing bag". For extra safety, if the nose-cone would leak some gas, we will have a continuous suction system collecting room air around the rat including any possible leak of 13N2 or isoflurane. All bags and tubes outside the PET-system will be covered /shielded by Lead to contain radiation from the experimenters/researchers.

The micro-PET scanner will be set to take a set of scans cycling through to image the entire body of the rat, over a course of 20 minutes.

Then ventilation will switch to rebreathing air including 13N2 (and isoflurane) for 30 or 45 minutes (with oxygen continuously added to keep normoxic gas at 21%O2). Then breathing will switch to inspiring regular air with isoflurane. Expired gas will be collected (containing isoflurane and 13N2). Radioactivity will be monitored on the inspiratory and expiratory side (inhaled and exhaled gas separated) as well as in body in 3 dimensions by the PET. All gas (isoflurane and radioactive nitrogen) will be collected. Upon completion of the scans, each rat will be euthanized, and the carcass kept until all activity has been allowed to decay away.
We expect this part to show that the radioactive gas goes into the rat, and that the signal can be detected appropriately by the PET. We also expect this part to develop calibration procedures, e.g. how many minutes scan time is necessary to yield images over certain body areas.
We will test 3 animals each at 30 and 45 minutes of breathing radioactive gas.

Study or Experiment Name	C	D	E
3. Pressure Chamber Development and gas rebreathing equipment testing	0	2	0

Briefly describe the scientific question or rationale to be addressed or, if not performing an experiment, the purpose served by use of animal subjects. Describe the study to be performed. This generally involves describing the experimental design including all experimental groups and controls, sample sizes, procedures and/or manipulations, timelines, and endpoints. If a procedure, surgery, or collection is performed multiple times, this must be described here. For each text box, provide a calculation of the total number of animals requested and identify the appropriate pain/distress category for the animals. Do not include procedural details here; procedures should be described in the sections below. Likewise describe in Question 13 how sample sizes are determined. A reviewer should be able to understand the complete time-course of procedures on each animal that you describe in the studies below.

We will be constructing and utilizing a specialized pressurization chamber. Not only will this chamber have to withstand the increased pressures of simulated diving, but it will also have to be radioactively shielded and equipped with reclamation devices to collect the radioactivity at the end, as well as an isoflurane vaporizer that can still push anesthetic against the increased pressures. We hope also to be able to build a chamber that can be inserted into both a PET and MR, enhancing safety, but a more difficult system to design and build. Using an acrylic tube will enable a PET-compatible chamber. It is unclear whether the MR signal gets distorted too much by such a chamber. If not compatible the animal will be imaged without the chamber in the MR and the images overlaid/morphed to get an anatomical map (correlation with PET). Most of these tests can be done without animals but the addition of a live animal (that has to be anesthetized) has to be incorporated into the development to ensure gas can be delivered to the rat in the chamber in a timely fashion before radioactivity decays. The MR signal is affected by the surroundings, i.e we can test the chamber with a “MR Phantom” but ultimately 1 or 2 animals needs to be tested as well.
We expect this part will lead to a fully developed pressure chamber that can be positioned inside a PET-MRT system with full imaging capabilities of an anesthetized rat during compression and decompression while breathing isoflurane with either the radioactive gas or gas equivalent to air (with collection of exhaled gas that partly contain anesthetics and in part radioactivity). The same animal used to test the rebreather/gas collection system and chamber can then be used (while still anesthetized as described in experiment 1) to test the MR and calibrate the MR acquisition. Anticipated duration of anesthesia will be 2-3 hours as in the experiments reported in experiment 1 above.

Study or Experiment Name	C	D	E
4. Impact of Exercise on Nitrogen Reservoirs in Pressurized Rats	0	25	0

Briefly describe the scientific question or rationale to be addressed or, if not performing an experiment, the purpose served by use of animal subjects. Describe the study to be performed. This generally involves describing the experimental design including all experimental groups and controls, sample sizes, procedures and/or manipulations, timelines, and endpoints. If a procedure, surgery, or collection is performed multiple times, this must be described here. For each text box, provide a calculation of the total number of animals requested and identify the appropriate pain/distress category for the animals. Do not include procedural details here; procedures should be described in the sections below. Likewise describe in Question 13 how sample sizes are determined. A reviewer should be able to understand the complete time-course of procedures on each animal that you describe in the studies below.

Each rat will be anesthetized as described in experiment 1-3. Once unconscious, the rat will be placed in the chamber and the anesthesia/radioactive gas line will be securely attached to their face via a sealed nosecone and rebreather device. The chamber will be sealed and pressurized while the animal remains asleep. Breathing of 13N2 will commence at the start of compression and last until decompression is complete. We will compress and decompress 10+10 rats (control plus pre-exercised as in experiment 1) in the chamber either directly in the PET (if such is constructed) or in a chamber positioned next to the PET, breathing 13N2. The microPET scanner (Vista DR, GE Healthcare), is located in (b) (6)(b) (6)(b) (6) (b) (6)
After decompression the rats will be imaged with PET and gas distribution measured during 1-2 hours of breathing air (all exhaled gas collected separately). Blood will NOT be sampled. The primary imaging data targets will be the brain and the pelvic area (with hips, large muscles etc.) which are areas that does not move in a sedated animal. PET will yield ventilation studies of the lungs in addition to imaging of stationary tissues. MR imaging will follow the same protocol as the PET imaging, by moving the whole chamber into the MR after radioactivity has decayed beyond what can generate images in the PET. The still anesthetized rats will be scanned to get good images of the relevant body areas, and total scan time will probably be about 20 minutes. We expect this experiment to be the proof of concept of studying gas in decompression, and also, show where the gas is distributed in the body and answer the hypothesis on gas diffusivity and changes in supersaturation tolerance. As in the other experiments' animals will be sacrificed after imaging is concluded.

Group Pain Category Subtotals and Totals	Subtotal C	Subtotal D	Subtotal E	Total(C+D+E)
	0	58	0	58

13. Justification of the number of animals requested. How were the sample size, number of groups, and number of repetitions determined?
Check all that apply.

- [ ] Power analyses indicate that the proposed number of experiments is the lowest required for statistically valid tests of the hypothesis. Describe below.
- [x] The experiments will compare the effects of several independent variables and therefore require many groups or cohorts. Describe below.
- [ ] The outcome measures or phenomena being measured are variable and large sample sizes are necessary for statistically valid sampling. Describe below.
- [ ] Differences from controls are expected to be small, and large sample sizes are necessary to distinguish differences reliably. Describe below.
- [x] The experiments are technically difficult and multiple attempts will be needed to obtain satisfactory data from each experiment. Describe below.
- [ ] These animals will be used to produce antibodies or tissues. Describe the amount of tissue needed and how much is produced from each animal below.
- [x] This is a pilot study to obtain preliminary data to determine if a larger study can be done. Describe below.
- [ ] This is product testing done under FDA guidelines which necessitate using this sample size. Describe below.

Other - Explain in detail below

The proposed project includes 2 separate experiments that need 20 animals each over 3 years (5-10 in each control vs intervention). It also needs a few animals to set up and validate/calibrate the radioactive gas technique, a new chamber design etc. Also including 10 animals as "backup" considering these are logistically challenging experiments we may need to run more than the expected number in each group to get complete data sets.

/. Experimental Details

Questions 14-29

14. Breeding of Animals

- [x] We will not do Breeding of Animals on this Protocol

15. Behavioral Studies

- [x] We will not do Behavioral Studies on this Protocol

16. In Vivo Blood/In Vivo Fluid/In Vivo Tissue Collection

- [x] We will not do In Vivo Blood/In Vivo Body Fluid/In Vivo Tissue Collection on this Protocol

17. Administration of Anesthetic, Analgesic, Therapeutic and Experimental Compounds

- [ ] We will not administer any compounds to animals on this protocol

Federal regulations require that all compounds administered to animals be pharmaceutical grade if commercially available, including diluents.
Please see:

- Policy on the Use of Non-Pharmaceutical Grade Compounds in Animals
- UCSD Recommended anesthetic and analgesic compounds by species
- Rodent anesthesia equipment

17 A. List all compounds that will be used in/on live animals. Click the Save button to save the current line and add more as necessary.

Experimental Compound

Compound	Dose	Route	Frequency and Duration
13N2	<1% of breathed air	Inhaled	Once, 1-2hours

Possible adverse effects:

None, 13N2 is chemically identical to normal nitrogen in air.

Anesthetic/Euthanasia Compound

Compound	Dose	Route	Frequency and Duration
isoflurane	2-5%induction 1-3%maintanence	inhaled	throughout the imaging part of the experiment.

A) Criteria for assessing depth of anesthesia and, if using inhalant anesthesia, B) description of apparatus for administering Compound (e.g., precision vaporizer):

reflexive extremity and eye movement and general body response, heart rate and respiration

Anesthetic/Euthanasia Compound

Compound	Dose	Route	Frequency and Duration
Sodium Pentobarbital	150 mg/kg	IV	once

A) Criteria for assessing depth of anesthesia and, if using inhalant anesthesia, B) description of apparatus for administering Compound (e.g., precision vaporizer):

followed by removal of a major organ (or bilateral thoracotomy).

17 B-C. Non-Pharmaceutical Grade Compounds
Federal regulations require that all compounds administered to animals be pharmaceutical grade if commercially available, including diluents.

Please read IACUC Policy 31 on the Use of Non-Pharmaceutical Grade Compounds in Animals before answering the following questions.

17 B. Pharmaceutical grade formulations are widely available for *therapeutic* compounds (anesthetics, analgesics, antibiotics, euthanasia compounds, etc.) that are not used experimentally.

Do you propose to use any *therapeutic* compound on animals that IS NOT pharmaceutical grade?

- ○ Yes
- ◉ No

If YES, name each non-pharmaceutical therapeutic compound from 17 A., provide scientific justification for not using a pharmaceutical grade product and describe how you will assure purity, sterility, and effectiveness of each preparation. If preparation methods vary per compound, please describe the methods to be used for each compound or class of compounds.

17 C. 1. Pharmaceutical grade compounds may or may not be available for *experimental* compounds (test compound, compounds used to induce a model or condition, tracers, etc.).

Do you propose to use any *experimental* compound that IS NOT pharmaceutical grade?

- ○ Yes
- ◉ No

If YES, name each non-pharmaceutical experimental compound from 17 A. and describe how you will assure the purity, sterility, and effectiveness of each of your preparations. If preparation methods vary per compound, please describe the methods to be used for each compound or class of compounds.

17 C. 2. Do any of the *experimental* compounds listed in 17 C. 1. have a pharmaceutical grade preparation that is commercially available?

- ○ Yes
- ◉ No

If YES, name each non-pharmaceutical grade compound from 17 C. 1. and provide scientific justification for not using the pharmaceutical grade preparation(s). If your justification varies per compound, please provide scientific justification for each compound or class of compounds. Example wording for scientific justification can be found in IACUC Policy 31.

18. Paralyzing Compounds
- ☑ We will not use Paralyzing Compounds on this Protocol

19. Surgery
- ☑ We will not do Surgery on this Protocol

20. Food or Water Restriction
- ☑ We will not do Food and Water Restriction on this Protocol

21. Extended Restraint
Do not include brief periods of restriction for blood collection or injection. Please read Policy 27 on Extended Restraint before answering the following questions:

- ☑ We will not do Extended Restraint on this Protocol

22. Tumors/Neoplasia
- ☑ We will not do Tumors and Neoplasia on this Protocol

23. Death as an Endpoint
- ☑ We will not do Death as an Endpoint on this Protocol

24. Arthritis
- ☑ We will not do Arthritis on this Protocol

25. Adjuvant Use or Polyclonal Antibody Production
- ☑ We will not do Adjuvant Use or Polyclonal Antibody Production on this Protocol

26. Monoclonal Antibody Production
- ☑ We will not do Monoclonal Antibody Production on this Protocol

27. Introduction/Injection of biologicals (tissue, cell lines, tumors, stem cells, blood components, body fluids) into rodents
- ☑ We will not use Biologicals in Rodents

28. Medical Imaging or Irradiation
- ☐ We will not do Imaging or Irradiation on this Protocol

What is the experimental group (from Question 12) which requires Imaging or Irradiation? Describe the procedure(s), including use of anesthesia or sedation, restraint used, contrast media administered, etc.

All animals will be anesthetized and breathe through a nose-cone supplying the breathing gas and the Isoflurane. This will commence before any compression/decompression or imaging starts. We will monitor breathing and the temperature continuously.

For animals breathing 13N2: PET imaging will be performed in each rat. Each rat will be anesthetized before being placed into the compression chamber. Once unconcious, the rat will be placed in the chamber and the anesthesia/radioactive gas line will be securely attached to their face via a sealed nosecone and rebreather device. The chamber will be sealed and pressurized while the animal remains asleep. Following the compression period, the rat will be kept at high pressure for a time and then the pressure will be released. The still unconcious rat will be placed in the microPET scanner (Vista DR, GE Healthcare), located (b) (6)(b) (6)(b) (6)(b) (6)(b) (6) [redacted] and then imaged for 90 minutes. They will will be under general anesthesia (isoflurane) the entire time. Blood will NOT be sampled. The study will require about 2.5 hours of anesthesia time and 90 minutes of ´in-scanner´ time. At the conclusion of scanning animals will be sacificed.

Animals undergoing ultrasound scans will be anesthetized prior to beginning the scan with isoflurane. They will be shaved and Naired over the heart, then will follow the same procedure of pressurization as the PET scan rats, although they will not receive radioactive nitrogen, instead receiving normal pressurized air. Following the compression cycle, they wil be loosely restrained on a warmed imaging stage, while still anesthetized. Warmed ultrasound gel will be used to couple the transducer to the animal over the chest, and imaging will occur over the course of 1 to 2 hours. Imaging modes may include normal B-mode, color doppler mode, or contrast mode. All modes utilize similar energy levels, and all are well below the threshold for mechanical or thermal tissue disruption. Total anesthetization time will be simialr to that for PET studies. Follwoing these scans, the animals will again be sacrificed.

MR imaging will follow the same protocol as the PET imaging, but without any use of contrast agents. Anesthetized rats will be scanned to get good images of the relevant body areas, and total scan time will probably be about 20 minutes.

List locations(s) of procedures(s) including building, room number and equipment used.

The PET and Ultrasound studies will be performed in (b) (6). MR studies will take place in (b) (6)

29. Other Procedures

Describe any procedures (such as non-surgical procedures, monitoring, measurements) to be performed on animals that are NOT discussed above.

Treadmill Exercise
Rats will be placed on a motorized treadmill for the exercise period. The treadmill will begin with a warm-up period of 20min at 0.2m/sec, before entering the interval period, when the rats will run at 0.36m/sec for 8min, followed by 0.2m/sec for 2min. These intervals will repeat until the animal has run for a total time of 1.5hrs.

The treadmill will force them to run according to set parameters (the speed of the treadmill run is published data. We will most likely use a commercially available treadmill with an electric grid at the rear of the treadmill to provide shocks if rats do not run willingly. Shocks will be no greater than 2mA. We will also consider systems with the option to use air puffs as an alternative encouragement mechanism. All rats will be observed directly during exercise to reduce intensity or finish the exercise bout if rats are unable to run (to avoid multiple shocks). This exercise protocol follows previously published data, however if we find that the rats we use are unable to reliably run for the established 1.5hr period, we will reduce the time, intensity, or both. After this exercise they will go back to their regular cage until the next day.

Pressurization
Anestetized rats will be put in the chamber at standard pressure (~100kPA). Once sealed inside, the pressure be increased at a rate of 100kPA/min until it reaches 700kPa (roughly 7atm) and remain at that pressure for either 30 or 45 minutes. At the conclusion of the diving period, pressure will be released slowly at a rate of 50kPA/min until the chamber returns to 100kPa.

/I. Potential Animal Pain and Distress/Euthanasia

Questions 30-34

30. Describe the potential study-induced problems the animals might experience (i.e. post-surgical, dehydration, weight loss, pain studies, use of aversive stimuli, extended restraint, retro-orbital bleeding, CFA/IFA injection, arthritis, tumor development, impaired ambulation, other induced disease). Include any health problems due to the phenotype of the animal.

Imaging itself presents no expected health risks. All these imaging procedures have been done many times before in this imaging facility.

The focus of this study is decompression sickness. Upon release of the increased pressure they are placed under, we expect nitrogen bubbles to form in the joints and blood of these animals. Decompression sickness symptoms include pain, dizziness, disorientation, and in extreme cases, such as those we are studying, potential death. Animals will be anesthetized (with monitoring of anesthesia level) the entire time that decompression sickness is possible, and animals will be euthanized under anesthesia, so they will not experience any of these effects.

Exercised animals may experience some distress from the running period. There will be an electrified grid or small air puffing gun at the rear of the treadmill to encourage the animals to run. Shocked from the grid will be minor and will cause no more discomfort than needed to get the animal running. Discomfort from the physical exertion will likely occur, but we do not plan to run any rat to exhaustion.

31. For each of the potential problems from Question 30, describe how any pain and/or distress will be recognized. Provide the specific clinical signs which will be monitored as well as the frequency of monitoring, including provisions for off hours.

Any movement, increase in heart rate or jaw tone, pupil reflex, or response to toe pinch will be taken as needed for additional anesthesia.
Each animal will be monitored continuously during the whole experiments from start of anesthesia until euthanasia.
Animals will be also be monitored for breathing and temperature.

Distress during exercise will be monitored by watching for exceptionally high breathing rates, as well as for signs of exhaustion, such as not continuing to run even when shocks or air puffs are provided as encouragement.

32. For each of the potential problems from Question 30, explain what steps will be taken to alleviate any pain, distress or discomfort the animals may experience (i.e. call ACP veterinary services, euthanasia, administer analgesia, etc.). If analgesics will not be used, please scientifically justify the reasons.

Increased anesthesia (isoflurane).

Animals showing signs of distress or exhaustion will have the intensity of their run reduced. If they do not recover with this reduction, exercise will be discontinued.

33. Describe in detail criteria for euthanasia:

Any animal exhibiting pain or distress shown by movement that cannot be alleviated by increased anesthetic will be euthanized. All animals will be euthanized at the end of imaging experiments.

☑ I concur that I will euthanize moribund animals immediately.

34. Method of Euthanasia The Institutional Animal Care and Use Committee policy at Policy 13 Euthanasia requires investigators to follow the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition unless scientific justification is provided below. Please include ALL methods of euthanasia. Describe the secondary physical method to be used in rodents that are euthanized by CO2.

Sodium Pentobarbital 150 mg/kg IV, or CO2 asphyxiation, this will be followed by removal of a major organ or bilateral thoracotomy.

/II. Animal Locations and Transport

Questions 35-39

35. Please indicate building(s) and room number(s) of research laboratories where live animals will be transported, housed or where animal procedures will be performed. Do not include vivaria or vivarium procedure rooms here.

Building:	(b) (6)(b) (6)(b) (6)(b) (6)	Room Number:	(b) (6)

Building:	(b) (6)(b) (6)(b) (6)(b) (6)	Room Number:	(b) (6)
Building:		Room Number:	

36. Transport of animals out of the animal housing facility (vivarium) is strongly discouraged and return trips to the vivarium are not only discouraged but prohibited for some animal populations. If animals must be transported out of the vivarium, indicate where animals will be taken, why animals are transported, what procedures are performed on animals in the laboratory, how animals will be transported, how often animals will be transported and whether or not animals will be returned to the vivarium. If you do not transport animals, please state N/A.

Animals will be transported by ACP in a micro isolation cages from the vivarium to the (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)

☑ I have read and agree to abide by the Policy 33 Transportation of Rodents.

37. Request for Satellite Housing. Housing animals outside of an approved ACP vivarium for greater than 12 hours is against UCSD Policy 28 . If you are requesting an exception to this policy, please indicate housing site requested and justify the need to do so. If you do not keep animals out of the vivarium for greater than 12 hours, please state N/A.

Animals to be exercised one day and imaged the next will remain in (b) (6) (a satellite facility) overnight, unless we receive permission to return them to their housing in (b) (6)

38. Standard housing, food and water, and enrichment is provided by ACP as described in Policy 12 - Housing and Environmental Enrichment for Laboratory Animals

☑ Check here if you require only standard ACP husbandry.

OR describe below if you require non-standard housing (such as single-housing of social species for experimental reasons), non-standard feed or water (such as food on the cage floor, specially formulated diets, additives to drinking water, addition of sunflower seeds or other food enrichment) or non-standard enrichment. Please provide scientific justification for single-housing of social species.

39. There are specific policies and guidelines for animal work that is funded through UCSD but performed away from the UCSD campus. Please read Policy 21 Inter-Institutional Research.

☑ Check here to indicate that you will perform all experimental work at UCSD campus locations.

If any animal work will be done at other locations, please describe in detail below. Include animal work that might be done at Contract Research Organizations (CROs) or collaborator's locations such as imaging, irradiation, housing, breeding, surgeries, euthanasia, tissue collection, etc. Include locations of custom antibody production (but do not include information for antibodies purchased "off the shelf"). Include surgery performed by the vendor prior to arrival at UCSD (e.g. ovariectomy, vasectomy, adrenalectomy, catheterizations, etc). Provide all information about the location(s) where this animal work is performed: company or institutional name, address, telephone number, PHS assurance number, and AAALAC accreditation date.

/III. Hazardous Agents

☐ This project DOES NOT include hazardous chemicals, microbial organisms, recombinant DNA, human cells, radioactive materials, or animals that carry zoonoses. If any hazardous agents are used in live animals, please describe below. A project that uses hazardous agents may not begin until the investigator has obtained any necessary EH&S BUA, RUA or appropriate IBC approval.

Agent:

-- Other hazard not listed

Specific Description of Hazard (strain, type, etc.)
Radioactive isotope of nitrogen gas. 13N.

How is Hazard used?
The positron emitting isotope 13N has a radioactivity with short time span. Half-life is 9.96minutes. The rats will breathe some of this gas instead of regular atmospheric nitrogen gas. Imaging will be done in a PET for as long as there is measurable activity (about 2-3 hours).

Reviewer: DS **Review Date:** 11/12/2019

Prescribe handling for research staff:

R4: High level radiation

- **Follow conditions of the RUA 720 at all times**
- **Follow Section 2.3 of the Radiation Safety Manual (Care and Handling of Animals Containing Radioactivity)**
- RUA holder is responsible for posting rooms, conducting routine surveys of cages and rooms, changing radioactive animal bedding, removing and disposing of all radioactive waste and carcasses, decontaminating cages and rooms and de-posting rooms
- Monthly contamination surveys for low use labs and weekly contamination survey for high use labs

- **Ensure proper labeling of all animal cages and rooms at all times**
- Do not handle unshielded sources directly
- Store behind lead shielding
- Use lead shielding to minimize exposure
- Extremity and whole body dosimetry required
- EH&S approved isotope administration, imaging and transportation procedures
- EH&S approved waste handling, storage and decay procedures
- PET specific lead shielding required
- Perform radiation exposure surveys during and contamination surveys after procedure

NOTE: wear lab coat, gloves, and safety glasses. To avoid cross contamination never wear gloves outside of the research lab

Prescribe handling for animal care staff:

- Prior to entering room an ACP employee must observe signage posted on the door (See "Typical Caution Radioactive Material" signs used at UCSD) and follow all instructions for proper PPE before entering the room.
- Put on appropriate PPE prior to entering the room as follows:
  - Lab coat
  - Gloves
  - Safety glasses

☐ Hazard Use has **NOT** been approved by EH&S
☐ Safety Considerations Meeting is Required

Agent:

Isoflurane

Specific Description of Hazard (strain, type, etc.)
Isoflurane 1-3%

How is Hazard used?
Anesthetic

Reviewer: DD **Review Date:** 11/12/2019

Prescribe handling for research staff:

For the safe use of anesthetic gas in research environments use:

blink.ucsd.edu/safety/research-lab/chemical/anesthetic.html

Use the following link to report a work-related injury, illness, or hazardous material exposure

http://blink.ucsd.edu/go/injuryreport

Prescribe handling for animal care staff:

For the safe use of anesthetic gas in research environments use:

blink.ucsd.edu/safety/research-lab/chemical/anesthetic.html

Use the following link to report a work-related injury, illness, or hazardous material exposure

http://blink.ucsd.edu/go/injuryreport

- [ ] Hazard Use has **NOT** been approved by EH&S
- [ ] Safety Considerations Meeting is Required

Reviewer: DD **Review Date:** 11/12/2019

Prescribe handling for research staff:

For information about and approval to use controlled substances visit

http://blink.ucsd.edu/safety/research-lab/controlled-substances/

And follow directions

For all injuries and incidents see

http://blink.ucsd.edu/go/injuryreport

Agent:

-- Other hazard not listed

Prescribe handling for animal care staff:

For information about and approval to use controlled substances visit

http://blink.ucsd.edu/safety/research-lab/controlled-substances/

And follow directions

For all injuries and incidents see

http://blink.ucsd.edu/go/injuryreport

Specific Description of Hazard (strain, type, etc.)
Sodium Pentobarbital

How is Hazard used?
Sodium pentobarb will be used as a euthanasia agent

- [ ] Hazard Use has **NOT** been approved by EH&S
- [ ] Safety Considerations Meeting is Required

X. Funding

Identify all funding for this project

Grant PI	UCSD Proposal #:(20xx-xxxx)	Covering Dates From (mm/dd/yy)
(b) (6) [redacted]	EPD Proposal# is 00023059	01/01/20 to 12/31/22

Agency	Grant Title
Office of Naval Research	Where is the gas? A new method to study nitrogen during diving and decompression

- [ ] Grant is extramural, competitive, and peer-reviewed
- [ ] Funds have been awarded
- [x] Funds Pending

Grant PI	UCSD Proposal #:(20xx-xxxx)	Covering Dates From (mm/dd/yy)
		to

Agency	Grant Title

Funds have been awarded

Funds Pending

Grant is extramural, competitive, and peer-reviewed

Provide additional funding information here:

(. Other regulatory approvals that you may need before beginning your project

☑ I have read all of the documents listed here which apply to my animal protocol

Are you using human embryonic stem cells?
You are responsible for knowing and following the federal, state, and UCSD regulations and obtaining approval from the UCSD Embryonic Stem Cell Research Oversight Committee. For more information go to the UCSD Stem Cell website.

Are you using human blood or other tissue in animals?
You are responsible for knowing federal, state and UCSD IRB regulations and may need to receive IRB approval prior to beginning your studies. Please read the information at the UCSD IRB website.

Is any of your funding from a non-federal source (including departmental funds, gifts, private grants, clinical trial agreements, lab service agreements, and for profit contracts)? Is anyone listed on your protocol an employee, officer, or stockholder of the funding source? Do you receive income from the funding source?
You are responsible for knowing and following the UCSD, state and federal regulations pertaining to Conflict of Interest. For information and application go to the Conflict of Interest website.

Will you use controlled substances as anesthetics, analgesics or test substances in this protocol?
You are responsible for knowing and following UCSD rules and you must obtain a CSUA. More information and forms at EH&S Controlled Substances Acquisition, Storage, and Disposal Requirements.

Are you using hazardous agents?
You must obtain any necessary BUA, RUA, or IBC approval before starting this project. You must train your laboratory personnel and key Animal Care Program personnel regarding safe handling, disposal and cleanup of the hazardous agent. You must assure that areas where hazardous agents are used have appropriate signage and necessary PPE available. More information and appropriate forms are available at Environment, Health and Safety website.

Will you use anesthetic gases in this protocol?
Follow the Safe Use of Anesthetic Gases in Research Environments guidelinesto control the risk of exposure to waste anesthetic gases.

Do you have employees or students that handle animals as a part of their job or training?
You must assure that each of your employees and students has submitted an online Personnel Qualifications form (PQ), that each person has been added to your protocol, and that each person has taken the Risk Assessment Questionnaire and been given the opportunity to participate in the Medical Surveillance program. For more information, go to the Occupational Health Policy website.

Are you using animals in teaching undergraduate, graduate or continuing education students?
You must educate students on the ethical use of animals in research, appropriate handling, and student health and safety concerns. You must post and/or distribute the following poster to students before exposure to animals: Student Health Flyer.

Will you use the UCSD Center for fMRI?
You are responsible for submitting an application to the fMRI Center Biomedical Applications Committee (fCBAC) at http://fmri.ucsd.edu.

Are you working with Dual Use Research Agents or performing Dual Use Research of Concern (DURC)?
You are responsible for reporting DURC agents used in research to the UCSD DURC Institutional Review Entity for DURC review through the BUA process. For more information on DURC agents, the experimental effects of concern and the DURC review and approval process go to the UCSD DURC website.

Do you have federal grants that are supporting this protocol?
If so, NIH requires that grants receive concomitant updates with protocol amendments in some cases. NIH states, " Grantees must also obtain prior approval from NIH for changes in scope, direction, or other areas that constitute a significant change from the aims, objectives, or purposes of the approved project. The grantee must make the initial determination of the significance of the change and should consult with a Grants Management Officer as necessary."

(I. Investigator's Assurance For the Humane Care and Use of Animals Used in Teaching and Research

☑ I am the Principal Investigator and I have thoroughly read, agree with, and will actively promote and enforce all of the assurances listed below:

1. I agree to abide by PHS Policy, USDA Regulations, UCSD policies for the care and use of animals, the provisions of the ILAR Guide to the Care and Use of Laboratory Animals, and all other federal, state, and local laws and regulations governing the use of animals in research.
2. I understand that emergency veterinary care will be administered to animals showing evidence of pain or illness, in addition to routine veterinary care as prescribed for individual species. I understand that it is my responsibility to provide current and updated emergency contact information for personnel who must be contacted in an animal emergency. I understand that any unanticipated pain or distress must be reported to the veterinarian or his/her designee.
3. I assure that I have consulted a veterinarian in the preparation of this proposal, if it includes procedures that could cause pain and distress to a vertebrate animal.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified biomedical scientist listed on this protocol.
5. I certify that all personnel having direct animal contact, including myself, have been trained in humane and scientifically acceptable procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.
6. I certify that all personnel in this project will attend Orientation to Animal Research and all mandatory classes as determined by each individuals Personnel Qualifications Form.
7. I understand that the use of hazardous agents in animals may only be initiated after approval from EH&S and I am responsible for complying with all safety related information stated under section VIII of the protocol.
8. I certify that all personnel working on this protocol will be given the opportunity to participate in the Medical Monitoring Program at the Center for Occupational and Environmental Medicine (COEM). All personnel on this protocol will be made aware of the hazards involving the use of live animals and tissues.
9. I understand that I must submit an amendment for any proposed changes to this protocol and wait for IACUC approval before beginning the work.

10. I understand that should I use the project described in this application as a basis for a proposal for funding (either extramural or intramural), it is my responsibility to ensure that the description of animal use in such funding proposals are identical in principle to that contained in this application.
11. I understand it is the responsibility of the Principal Investigator to ensure the safe and ethical conduct of all research conducted under this protocol, and to assure that all research is carried out following federal, state, local, and UCSD policies governing animal research.
12. I certify that I will maintain complete, up-to-date and accessible records of procedures on animals as required by policy and regulation.
13. I declare that the information provided in the accompanying protocol is accurate to the best of my knowledge.
14. I certify that all state, federal and international permits for the use of the animals described in this protocol are in place (or will be in place before studies begin) including those permits mandated by the Department of Commerce, Marine Mammal Protection Act, Bureau of Land Management, National Forest Service, and foreign countries.
15. I acknowledge that clicking on the "submit protocol" button below is equivalent to an electronic signature.

UC San Diego (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
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UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

RESEARCH INTEGRITY & COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	(b) (6)
FROM:	(b) (6) (b) (6)(b) (6)(b) (6)(b) (6)(b) (6) Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	2/12/2020
PROJECT TITLE:	Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels
FUNDING SOURCE:	Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.) Office of Naval Research
IACUC PROTOCOL #:	R IS00007438
PROTOCOL STATUS:	**Procedural Change (Verified)**

Your Modification concerning the above referenced IACUC protocol was reviewed by the Veterinarian Verification and Consultation process on **2/12/2020** for the following changes:

Modifications: Characteristics of Animals and Pain Category of Research 5.1.1

Changed: Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)

New Value: 305

Old Value: 153

Modifications: Statistical Analysis and Rationale 9.1

Changed: Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)

New Value: 305

Old Value: 153

Modifications: Statistical Analysis and Rationale 9.1.1

GRAND TOTAL OF RATS NEEDED FOR 3 YEARS: 305 + 122 + 13 + 12 = 452 Sprague-Dawley rats of various ages

Section 5.1.1., 9.1, 9.1.1.
I made a math error in the original protocol when I added up the total number of Sprague-Dawley rats needed for MitoQ dosing, HBO divers & radio telemetry experiments. The correct number is 305 and the wrong number listed is 153. The new higher total comes from Experiments Nos. 1-5 in the protocol with the following n-values (Expt. No. 1 has two parts): 62 + 20 +40 +45 +30 + 78 + 30 = 305

The total number of rats needed are 452.

Section 9.1.1. I completed a calculation for the animal number that had been omitted as a cut and paste error from another section of the protocol. The 'n' value was correct; the calculation was incompletely listed.

BUMED Animal Use Appendix for Research Involving Animals
ABBREVIATED VERSION

*****NOTE: BUMED will ONLY review protocols that have been approved by your IACUC.*****
****Animal work MAY NOT be initiated until the awardee receives BUMED approval.****
Animal work initiated without BUMED approval is noncompliant and may not be funded.

Institutions using DoD funds to support the use of animals in research, product development, testing and education projects must provide electronic copies of the following documents to the Department of the Navy (DON) Bureau of Medicine and Surgery (BUMED) Veterinary Affairs Office for review and approval prior to initiation:

a. A copy of their **IACUC-approved institutional protocol(s)** (BUMED will ONLY review **approved** protocols) and documentation of IACUC approval

b. A copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by BUMED **PRIOR** to implementation)

c. A completed Appendix for each IACUC-approved protocol.

This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs.

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DoD regulations. The DoD policies and requirements for the use of animals in DoD-supported research, development, testing and evaluation are described in DoD Instruction 3216.01, dated September 13, 2010 and SECNAVINST 3900.38C, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to BUMED Veterinary Affairs:

Phone: [redacted] (b) (6)
Email: DON – VRPP, [redacted] (b) (6)
Mail: Department of the Navy Bureau of Medicine and Surgery
ATTN: Director for Veterinary Affairs
Bldg 1564 Room 122
1564 Freedman Drive
Fort Detrick, MD 21702

~~~~~~

Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text. Submit electronic copies of the appendix only; please do not submit printed copies to BUMED. Any section of the Appendix that is not applicable to your proposal, e.g., no surgery or no prolonged restraint, should be marked "**No**" or "**N/A**". There are no space limitations for the responses.

It is essential that only animal studies or procedures documented in an IACUC–approved protocol or amendment be performed at your facility. BUMED will collect animal usage information for end-of-year DoD animal use reports separate from the grant annual progress reporting requirement throughout the life of your award. For this reason, Principal Investigators or other delegated research personnel should keep accurate records and be able to provide an audit trail of all animal use that correlates to their approved protocol.
~~~~~~

[X] Yes [ ] No All animal studies described in the attached IACUC-approved protocol are funded by this DOD award/contract. If no, STOP ! You must use the **full version** of the Animal Use Appendix.

1. Administrative Data: (Provide Attending Vet, IACUC, and Research Office info for the work site.)

DoD/Contract PI Name: Heath G. Gasier, PhD

Grant/Contract PI Email: (b) (6) **Phone:** (b) (6)

Protocol PI Name: Heath G. Gasier, PhD

Protocol PI Email: (b) (6) **Phone:** (b) (6)

Animal Research Site (RS): Duke University

RS Attending Veterinarian: (b) (6)

Attending Vet Email: (b) (6) **Phone:** (b) (6)

RS IACUC Point of Contact (POC): (b) (6)

IACUC POC Email: (b) (6) **Phone:** (b) (6)

RS Grants Office Point of Contact (POC): (b) (6)

Grants Office POC Email: (b) (6) **Phone:** (b) (6)

Animal Protocol Title: The role of GABA in susceptibility to oxygen toxicity with repeated HBO_2 exposure

2. Total Number of Animals Used (by Species) and USDA Pain/Distress Category :

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Mus musculus	C	96
Mus musculus	D	64
Mus musculus	E	432

USDA Pain/Distress Category Definitions:

- ***Column B:*** *Animals being bred, and animals being held for use in research, testing, teaching, experiments or surgery but not yet used for those purposes.*
- ***Column C:*** *List the number of animals that will experience no more than slight or momentary pain or distress as a result of experimental manipulations or procedures on this protocol.*
- ***Column D:*** *List the number of animals that will potentially experience more than momentary or slight pain or distress that **WILL** be alleviated through the use of anesthetics and/or analgesics.*

- ***Column E:*** *List the number of animals that will experience more than momentary or slight pain or distress that* ***WILL NOT*** *be alleviated or relieved with anesthetics or analgesics.* **If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:**. .

3. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals. Note the DoD-specific database requirements in subparagraph a.

a. Literature Source(s) Searched:

http://www.ntis.gov/products/fedrip.aspxhttp://projectreporter.nih.gov/reporter.cfm

☐ Federal Research in Progress (FEDRIP) http://www.ntis.gov/products/fedrip.aspx

or

☒ Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER) http://projectreporter.nih.gov/reporter.cfm

b. Date of Search:

c. Years Covered By Search:

1987-2021

d. Key Words and/or Search Strategy:

Alternatives to hyperbaric oxygen seizures; alternatives to animal models of hyperbaric oxygen seizures

e. Results of Search:

For "alternatives to hyperbaric oxygen seizures," 6 citations were found using PubMed and 4 citations were found using Web of Science. The projects varied in scope and are not related to this research. For "alternatives to animal models of hyperbaric oxygen seizures," no matches were identified.

****Questions 4-10 refer to the Research Site and Protocol Principal Investigator.****

Information and/or documents required in questions 4-8 should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to BUMED by the Protocol Principal Investigator (PI) or institution staffs. If the Protocol PI prefers that BUMED staff contact the institution to obtain this information and/or documents, the PI must specifically request this action in writing. The Protocol PI should contact BUMED (see contact information on cover page) to arrange for submission of this written request.

4. Institutional Animal Care and Use Committee(s) (IACUC) Approval(s):

Institutional Animal Use Protocol Number:	A224-21-11		
IACUC (initial) approval date:	Nov 19, 2021	Protocol expiration (rewrite) date:	Oct 31, 2024

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

While Duke employs multiple veterinarians, the management of veterinary medicine, animal care, and facility operations is retained by the Director, Division of Laboratory Animal Resources. Duke accepts the American College of Laboratory Animal Medicine's (ACLAM) "Guidelines for Adequate Veterinary Care" (ACLAM 1996) as a core reference document. These guidelines include expectations for

veterinary access to all animals and their medical records, regular veterinary visits to facilities where animals are or may be housed or used, provisions for appropriate and competent clinical, preventive, and emergency veterinary care, and a system for legal animal procurement and transportation. The Division of Laboratory Animal Resources (DLAR) provides husbandry and veterinary care to centralized vivarium operations; husbandry to select satellite facilities, and veterinary care to all satellite and PI-managed facilities. DLAR has a total of six full-time veterinarians, all are 100% FTE. Duke requires that emergency, weekend, and holiday care of animals is performed by qualified personnel. Emergency veterinary care is available after work hours, on weekends, and on holidays. In the event of an emergency, institutional security personnel and fire or police officials are able to reach people responsible for the animals. Notification is enhanced by prominently posting emergency procedures, names, or telephone numbers in animal facilities and by placing them in the security department or telephone center. Emergency procedures for handling special facilities or operations are prominently posted and personnel trained in emergency procedures for these areas. Specifically, DLAR regular animal care staff provide weekend and holiday care in core vivaria, while certified PI staff provide weekend and holiday care in PI-Managed vivaria. Duties covered on weekends and holidays include:

- Mortality-morbidity check
- Food and water check
- Change caging when excessively soiled or wet
- Environment check in all animal rooms
- Medical treatments as prescribed

A Veterinarian and Veterinary Technician (VT) are on call for weekend and holiday coverage. They are responsible for all scheduled treatment procedures during this period along with the VT. The VT performs most scheduled rodent treatments. Key personnel (Veterinarian, Veterinary Technician and Supervisor of Operations Manager) communicate during weekend and holiday periods via pagers and cell phones. A disaster plan that takes into account both personnel and animals has been prepared as part of the overall safety plan for the animal facility. The colony manager or veterinarian responsible for the animals is a member of the appropriate safety committee at Duke, an “official responder” in the institution, and a participant in the response to a disaster. Duke’s disaster plan (DP) covers acts of nature, terrorism, and other catastrophic events. The DP has been developed within Duke and approved by the IO and senior management. The plan takes into account both personnel and animals as part of the overall safety plan for the animal facility. The Director, DLAR is a member of the safety committee, an “official responder” at Duke, and a participant in the response to a disaster. As part of this plan, the Duke police have been involved in the planning and review of the disaster plan.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

☐ Yes ☒ No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

☐ Yes ☒ No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

☐ Yes ☒ No Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

(b) (6)	(b) (6) March 18, 2022
(Protocol Principal Investigator Printed Name)	(Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

Duke University
Institutional Animal Care and Use Committee
Approval Letter

Office of Animal Welfare Assurance
Campus Mail: (b) (6)
US Mail: (b) (6)

Voice (b) (6)
Fax: 919.668.6725
https://dacup.duke.edu/

November 19, 2021

RE: **Protocol Registry Number** A224-21-11; **Protocol Title** *The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure*

Dear Dr. Heath Gasier,

On November 18, 2021 the Duke Institutional Animal Care and Use Committee (IACUC) reviewed and **approved** the above referenced protocol.

The terms of this approval are as follows:

- **Approval Period:** This protocol is approved through **October 31, 2024** unless otherwise terminated. If this protocol will continue beyond this approval period, the Duke IACUC will require a complete review by Full Committee Review or Designated Member Review and approval prior reaching the expiration date indicated.
- **Continuing Review(s):** Annual IACUC reviews will be performed if this protocol meets specific criteria that requires an annual review by the Duke IACUC or upon request. In addition, the IACUC can re-review any/all components of this protocol at any time. Reference: Protocol Management (New Protocols, Amendments, and Continuing Review).
- **Principal Investigator Responsibilities:** Use of animals must be in accordance with all applicable federal, funding, local, and institutional regulatory and oversight requirements. This includes but is not limited to the Animal Welfare Act/Regulations, Public Health Services Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, the NIH Grants Policy Statement, VHA Handbook, the DOD Animal Care and Use Review Office requirements, AAALAC International, and Duke Animal Use Policies and SOPs. Please note, certain granting agencies (e.g. Department of Defense) also require their review and approval of the original protocol and any amendments prior to performing the animal work.
- **Approved Number of Animals:** You are responsible for using the minimum number of animals required to obtain valid results. You are required to track and document animal use by utilizing the processes established by the Duke Animal Care and Use Program. Reference: Animal Number Justification and Tracking and associated SOP(s).
- **Protocol Personnel:** All personnel working with animals under this protocol must be appropriately qualified and experienced to perform their role in the animal activity. Reference: Training and Continuing Education for Animal Researchers and Care Staff.
- **Exemptions or departures:** IACUC approved exceptions, exemptions, and/or departures to animal welfare standards must only be performed as described in the approved IACUC protocol.
- **Modifications/Amendments:** Animal activities can only be performed as described in the approved IACUC protocol. New animal activities or other protocol modifications cannot be initiated until final IACUC approval is obtained (e.g., amendment).
- **Reporting requirement:** All unforeseen, adverse, non-compliant, and animal welfare concerns/events should be reported as outlined in the policy IACUC Notifications.
- **Post-approval monitoring:** Post-approval monitoring is required for all IACUC approved animal activities at Duke. More information on post-approval monitoring can be found in the policy Post-approval Monitoring.

Visit the animal program website for links to all the resources indicated, updates, and additional information (https://dacup.duke.edu/). For questions please contact the Duke Office of Animal Welfare Assurance 919-668-6720; IACUC@Duke.edu.

Please do not hesitate to contact us if there is anything that we can do to facilitate your research.

Sincerely,

(b) (6)

Duke University Institutional Animal Care & Use Committee
Application for Animal Use

Protocol Registry Number: (Assigned by OAWA) A224-21-11

Previous Protocol Number: (Renewals Only): A023-19-01

PART I: CORE INFORMATION (Sections A - G required for all protocols)
SECTION A: ADMINISTRATIVE

Submission Notes:
* The required submission process is from the PI's 'duke.edu' e-mail address
  (no signatures required if submitted via the PI's 'duke.edu' e-mail address; or if a senior lab member submits from their 'duke.edu' e-mail address and adds the PI to the e-mail as a 'cc').
* Submit all documents directly to IACUC@DUKE.EDU
* Hard copies, scanned hard copies, and FAXed copies of program applications are not accepted.

A1. PERSONNEL INFORMATION

Principal Investigator: Heath Gasier Duke Unique ID: (b) (6)

Dept/Div: Anesthesiology/GVT Office Phone: (b) (6) Cell Phone: (b) (6)

E-mail Address: (b) (6) Campus Box: (b) (6)

Sponsor: (required when non-Duke faculty or Duke non-faculty PI): Duke Unique ID:

Dept/Div: Office Phone: Cell Phone:

E-mail Address: Campus Box:

Protocol Title: (for this proposal) The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

Grant Title: (if different from protocol) Oxidative tissue damage after HBO2 exposure using selected FDA-approved anti-epileptic drugs (AEDs)

Grant P.I.: (if different from protocol)

Funding Source: Office of Naval Research

Is grant congruency certification required?
(●) NO ( ) YES (then submit grant with protocol)

If funded, please provide the SPS #: 3130826

If funded, please provide the Grant #: N00014-18-1-2702

A2. EMERGENCY CONTACTS

( ) None. This is a field observation study. No animal holding, housing, management, or control will occur under this protocol.

(●) This is a study where animals will be held, housed, managed, or controlled.

In case animals are observed in distress, the following persons should be contacted:

Primary person to call In case of animal emergencies: (b) (6) [redacted] Office Phone: (b) (6) [redacted]

Home Phone: _____ Cell Phone: (b) (6) [redacted] Pager: _____

Alternative person to call In case of animal emergencies: Heath Gasier Office Phone: (b) (6) [redacted]

Home Phone: _____ Cell Phone: (b) (6) [redacted] Pager: _____

Do you have any special instructions to the veterinary staff concerning animal care practices or prohibitions in emergency situations involving animals under this protocol?

- (●) No. Veterinarians should use professional judgement.
- ( ) Yes. As described in Section S: Special Instructions for Emergency Animal Care.

A3. ROLE DELINEATION

NOTE: A Personnel Qualification Form (Part I: Section G) must be completed for every member of the research group listed on this protocol. The form should illustrate skills or training necessary for the roles specified below (e.g., surgeon, anesthetist, phlebotomist, breeder, provides husbandry, observer, etc.).

	NAME	UNIQUE ID	ANIMAL HANDLING ROLE	ROLE IN PROJECT
Add Name	Heath Gasier	(b) (6)	(●) YES ( ) NO	PI (handling, IP injections, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name	(b) (6) [redacted]		( ) YES (●) NO	Co-I (pathological examination of tissues)
Add Name	(b) (6) [redacted]	[redacted]	(●) YES ( ) NO	LRA I (animal welfare, handling, IP injections, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name	(b) (6) [redacted]	[redacted]	(●) YES ( ) NO	Pulmonary Fellow (handling, IP injections, seizure monitoring, euthanasia, tissue collection)
Add Name	(b) (6) [redacted]	[redacted]	(●) YES ( ) NO	RT II (handling, seizure monitoring, involuntary running, euthanasia, tissue collection)
Add Name	(b) (6) [redacted]	[redacted]	(●) YES ( ) NO	LRA I (animal welfare)
	DLAR Veterinary Staff (Personnel list maintained in DLAR)	N/A	YES	Provision of controlled substances as described on this protocol, for anesthesia, analgesia, sedation, euthanasia.

A4. COLLABORATING (INCLUDES SUB-CONTRACTING) INSTITUTIONS

Will any facilities other than Duke University (main campus, lemur center, or marine laboratory) or Duke University Medical Center be used for animal use activities (e.g., housing, experimentation, observation, or procedures)?

- (●) No. All work will be performed in Duke University or Durham VAMC facilities.
- ( ) Yes. Work will be performed at another institution.

A5. RENEWAL PROTOCOLS

Does this application renew an existing protocol?

( ) No. This is a new protocol. (●) Yes. This is a renewal protocol. Please complete items `A' and `B' below:

A. Briefly summarize (<150 words) the findings to date as they relate to the use of animals in the retiring protocol:

(b) (4) [redacted]

(b) (4)

[redacted]

B. Specify the numbers of animals (by species) that were used In the retiring study (include animals used during the full life of the retiring protocol - generally the last 3 years):

Mouse: Authorized 360
Used 304 Granite = 248 received, 56 on order

C. Please describe any adverse events that occurred with animals on the retiring protocol (if none, please state 'NONE'):

NONE

A6. IMPORTING & EXPORTING ANIMALS, TISSUES, OR ACTIVITIES

Will you be EXPORTING animals, specimens, samples (tissues, blood, organ, etc.), or research products outside of the USA?

- (●) No
- ( ) Yes. I commit to contact Duke Export Controls at 919.684.3030 prior to exporting any items. The items I am exporting include:

Will you be IMPORTING animals, specimens, samples (tissues, blood, organ, etc.), or research products outside of the USA?

- (●) No
- ( ) Yes. I commit to contact Duke Export Controls at 919.684.3030 prior to importing any items. The items I am importing include:

A7. MERIT REVIEW

Has this proposal received a scientific merit review?

- ( ) No
- ( ) Yes, this proposal is NIH funded, so an NIH Study Section (or similar) has provided (or will provide) a scientific merit review.
- (●) Yes, this proposal is federally funded (other than NIH), and that agency will provide a scientific merit review.
- ( ) Yes, this proposal is not federally funded. The funding agency has provided a scientific merit review.
- ( ) Yes, this proposal is departmentally funded, and the department has provided a scientific merit review.

The individual who performed the merit review was:

A8. SUPPLEMENTAL SECTIONS INCLUDED IN THIS APPLICATION

- [ ] Section H: Hazardous Agent Use
- [x] Section I: Transportation
- [ ] Section J: Chemical Restraint
- [ ] Section K: Survival Surgical Procedures
- [ ] Section L: Non-Survival Surgical Procedures
- [x] Section M: Non-surgical Procedures

- ☐ Section N: Field Capture / Field Studies
- ☐ Section O: Animal Use in Human Patient Care Areas
- ☒ Section P: Genetically Engineered Animal Use (No Breeding)
- ☐ Section Q: Breeding colonies (Including Genetically Engineered Animals)
- ☐ Section R: Antibody Production
- ☐ Section S: Special Instructions for Emergency Animal Care
- ☒ Section T: PI Managed Animal Facility (holding longer than 12 hours)
- ☐ Section U: Exemptions to The Guide or Routine Animal Care Procedures
- ☐ Section V: Working at the Durham Veterans Administration Medical Center (Duke MOU and VA Protocol Required)
- ☐ Section W: Working at the Duke Lemur Center
- ☐ Section Y: Aquatics, Amphibians, & Reptiles

SECTION B: ANIMAL USE JUSTIFICATION

B1. PURPOSE

Describe in lay terms the purpose of this animal use study (250 words or less):

It is known that increasing the GABA-to-glutamate ratio delays hyperbaric oxygen (HBO2) seizure latency, and this can be accomplished by administering FDA approved antiepileptic drugs (AEDs). We have determined in mice that a single 30 or 60-minute exposure to HBO2 at 5 ATA causes DNA oxidation, inflammation and selective degradation of mitochondria, i.e., mitophagy, mainly in the cerebellum and hippocampus. This is followed by mitochondrial biogenesis and injury resolution within 72-hours. The GABAergic AED tiagabine attenuates acute brain injury and enhances mitochondrial biogenesis. Because exposure to HBO2 increases sensitivity to central nervous system oxygen toxicity (CNS-OT), which is linked to oxidative lung injury, (b) (4) [redacted]

B2. BENEFIT

Describe the potential scientific benefit of the proposed study with respect to human or animal health, the advancement of knowledge, or the good of society (250 words or less):

HBO2 increases the partial pressure of oxygen (PO2) in the lungs, blood and tissues. The pulmonary and central nervous systems are very sensitive to changes in PO2, and high levels can cause oxidative tissue damage that can be lethal. The USN established single-depth oxygen exposure limits from a body of research conducted in the 1970's. The exposure limits serve to reduce the probability of developing CNS-OT that may culminate in tonic-clonic seizures, thus are restrictive for depth and time. This dive schedule constrains military diving capabilities, and may need to be exceeded during operations placing divers at increased risk. For this reason, research has been directed towards understanding the mechanisms of CNS-OT with the goal of finding targeted prevention strategies. This research may lead to new guidelines for the safe use of oxygen during diving operations. This work will also increase our knowledge of the mechanisms of HBO2 seizures and lung injury in the following ways:
(b) (4) [redacted]

B3. LITERATURE SEARCH FOR ALTERNATIVES TO POTENTIALLY PAINFUL PROCEDURES OR DISTRESSFUL CONDITIONS

NOTE: The Duke University Medical Center Library has provided a guide "Animals in Research: Searching Alternatives." The IACUC strongly recommends use of this guide to select relevant databases and to develop search strategies for alternatives to painful procedures/distressful conditions affecting animal welfare during the performance of this study.
The link is: http://guides.library.duke.edu/animal_alternatives
For personal assistance, please contact the Biomedical Research Liaison Librarian at iacuc-librarian@duke.edu.

Does the study include activities, procedures, or states of welfare that have the potential for producing pain or distress? Examples: surgical procedures, spontaneous disease, genotype induced disease, tumor development, chronic conditions adversely affecting welfare?

- ○ No. There is no potential for pain/distress.
- ◉ Yes. There is potential for pain/distress.

What are the potentially painful activities or distressful conditions proposed in this animal use application?
(Examples: surgery, toxins, ionizing radiation, implants, tumor development, adjuvants, sepsis, infectious challenge, chronic noise, heat, cold, stressors) Seizures in conscious mice.

A literature search for alternatives to the potentially painful/distressful conditions (listed above) was performed. The details are:

Date (day, month, year) literature search was performed: Oct 5, 2021

Years Covered By The Search (From - To): 1987-2021

Search Strategy / Keywords used in the search:
PubMed
=alternatives to hyperbaric oxygen seizures (6 citations)
=alternatives to animal models of hyperbaric oxygen seizures (0 citations)
Web of Science
=alternatives to hyperbaric oxygen seizures (4 citations)
=alternatives to animal models of hyperbaric oxygen seizures (0 citations)

Databases Searched (**Minimum of two <2> databases required. Check all databases searched.**):

☐ AGRICOLA Data Base	☐ Altweb
☐ AltBib	☐ SCOPUS
☒ PubMed/MEDLINE (is considered one database)	☐ ToxNet
☒ Web of Science	☐ Biosis
☐ Animal Welfare Information Center (AWIC)	☐ Zoological Record
☐ Other (define):	☐ Other (define):

Did the literature search reveal less painful/distressful alternatives to the potentially painful/distressful conditions that are proposed?

- ◉ No alternatives were found.
- ○ Yes, but they are not suitable alternatives for the following reason(s):

	Potentially Painful/Distressful Conditions In This Proposal (match to keywords used)	Write a BRIEF Explanation why the alternatives found to this potentially painful/distressful conditions were not acceptable alternatives.
Add Procedure	Seizures	One citation reported an in vitro model using rat aortic rings, not applicable to this research. The remainder relied on in vivo HBO2 exposures.

B4. LOCATION OF ANIMAL HEALTH / WELL-BEING RECORDS (not research data records)

Records documenting observation of animal health and well-being:

☐ Will not be kept by the research staff. State reason: _____

☐ Will be kept with the animal (next to or near the housing location).

☒ Will be kept at the following location: (b) (6) [redacted] (b) (6) [redacted]

B5. ANIMAL JUSTIFICATION (applies to all animals)

The justification for using live vertebrate animals rather than alternative means of achieving the research goal is: (check all that apply.)

☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems because:

Our studies are directed towards understanding how HBO2 causes oxidative tissue injury and requires an intact physiological system. No non-animal alternative meets these requirements.

☐ There is not enough information known about the processes being studied to design nonliving models. Explain:

☐ Other (explain):

B6. SPECIES JUSTIFICATION (address each species individually)

Species: Mus musculus

This species was selected for the study because of the following attributes (select all that apply):

☒ A large database exists allowing comparisons with previous data.

Explain: There is a large body of published data using mice in HBO2 as an experimental model for studying oxygen toxicity. The sophistication of genetic tools and the relative ease of mice breeding and housing allows for more accurate and repeatable experiments to determine the cause of HBO2 oxidative tissue damage.

☐ The anatomy or physiology is uniquely suited to the study proposed.

☒ This is the lowest species on the phylogenetic scale that is suitable for the proposed study.

Explain: This is the lowest mammal on the phylogenetic scale that can be used in our project when conscious animals will be used and invasive devices (catheters, electrodes and microdialysis probes) are not needed.

☐ Other attributes. *(details required)*.

Add Species

B7. NUMBER JUSTIFICATION (address each species individually)

Species: Mus musculus

The number of animals requested for this protocol is based on the following (select all that apply):

☒ A statistical estimate of the number required to achieve statistical significance.

Explain: Based on our laboratory's extensive experience, we have found that in HBO2 experiments with conscious mice, there is variability in CNS-OT onset and degree of oxidative tissue damage. In addition, we have traditionally used male mice only, however, this research will examine gender differences because all military occupations are now open to females. Are knowledge of female physiological responses to HBO2 is limited. In order to complete this research, 12 male and 12 female mice are required for each set of experimental paramaters.

☐ The estimated minimum number necessary to achieve the goals of the study in the absence of a statistical estimate.

☐ The number necessary to obtain sufficient tissue or other material for testing or analysis.

☐ The number required to provide sufficient technical training or practice for the number of trainees expected.

☐ The expected or established mortality associated with this procedure.

☐ Other *(details required)*.

Explain:

Add Species

B8. ANIMAL NUMBERS

Adult and/or juvenile (post-weaning) animals only:

NOTE: If this is a renewal protocol and there are animals remaining on the expiring protocol, the number of animals remaining on the expiring protocol (and transferring to the new protocol) must be included in the number of animals requested under the new protocol. Animals on the expiring protocol will be transferred to the new protocol upon activation of the new protocol.

Species: Mus musculus Age or weight range: 2-4 months Number required for 3-year protocol: 592

Distribution of above 3-year number of animals over USDA Categories: See Duke Policy 'Categories of Pain and Distress'

Category B: (Breeding animals)	Category C: (Non-Painful Procedures)	Category D: (Procedures using anesthesia/analgesia)	Category E: (Painful or distressful procedures without anesthesia/analgesia)
	96	64	432

Source of the animals:

NOTE: If transferred from another Duke protocol, provide PI name and protocol number. Jackson Laboratory/Duke Breeding & Gnotobiotic Core

Add Species

Will embryonic and/or pre-weaning/fledgling animals be used at any time during this protocol? See Duke Policy `Counting Animals`

- (●) No. Only post-weaning animals will be used.
- ( ) Yes. Embryos and/or pre-weaning/fledgling animals will be used.

B9. ANIMAL IDENTIFICATION (Applies to all animals. See Duke's Policy on Animal Identification for more details)

- ( ) Animal identification is not necessary for this protocol.
- (●) Animal Identification is necessary for this protocol.

Specify method(s) below (check all that apply):

- [x] Ear Tagging with a unique numbered tag
- [ ] SQ Radio Tag
- [ ] Collar
- [ ] Tattoo
- [x] Temporary Dye / Ink Marking
- [ ] Ear Punch or notch
- [ ] Toe clipping. (Please review the IACUC policy for toe clipping.)
- [ ] Other Methods:

B10. OVERVIEW OF EXPERIMENTAL DESIGN AND ANIMAL USE TIMELINES

Provide a summary of the overall experimental design. The description should define animal groups, group sizes, anticipated or established mortality for these procedures, and how each group will be tested or used. This section should not include a detailed review of surgery or other activities, but should include the use of any unique drugs or practices:

There will be three independent experiments that employ different experimental designs.

Experiment 1: (b) (4) [redacted] Male (n = 96) and female (n = 96) C57BL/6J mice (Jackson Laboratory) aged 8-12 weeks old will be used in this experiment. There will be three dive profiles. In profile 1, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min. In profile 2, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min and again 48 hours later. In profile 3, mice (n = 48) will be exposed to HBO2 at 5 ATA for 60 min and again 72 hours later. Forty-eight mice will serve as controls and breathe room air. Mice will be pre-treated (IP injection) 30 min before each HBO2 exposure with TGB (9.4 mg/kg, n = 96) or vehicle (0.9% NaCl, n = 96). Conscious mice are continuously observed during HBO2 for neuromotor convulsions, marking seizure onset.

Category C: 48

Category E: 144

Experiment 2: (b) (4) [redacted]. Male (n = 168) and female (n = 168) (b) (4) [redacted] aged 8-12 weeks old will be used in this experiment. (b) (4) [redacted]

(b) (4) are produced through (b) (4) [redacted] of C57BL/6 background. These mice will be created by the Duke Rodent Genetic Engineering Services over 6-7-months. Chimeras will be transferred to the DLAR Breeding Core (b) (4) [redacted] to cross the mice with a Flp-deleter line. Once Frt deletion is confirmed, homozygous mito-QC mice will be bred over 6-12-months and transferred in batches to the Division of Lab Animal Resources. The dive profiles and treatment will be the same as in experiment 1, and n = 96 will complete each dive profile. Forty-eight mice will serve as controls and breathe room air. Mice will be pre-treated (IP injection) 30 min before each HBO2 exposure with TGB (9.4 mg/kg, n = 168) or vehicle (0.9% NaCl, n = 168). Conscious mice are continuously observed during HBO2 for neuromotor convulsions, marking seizure onset.
Category C: 48
Category E: 288

Experiment 3: (b) (4) [redacted] Male (n = 32) and female (n = 32) C57BL/6J mice (Jackson Laboratory) aged 8-12 weeks old will be used in experiment 3. Mice will be habituated to a motorized rodent treadmill (Columbus Instruments) before completing a maximal aerobic capacity ($\dot{V}O_2max$) test. The treadmill will be located in CR II, room 0594CB. The treadmill contains a shock grid at the back of the running lane. The shock stimulus will be 0.2 mA, 200 ms pulses, 1 Hz. Habituation will consist of four 10-min running sessions at 0, 3, 6 and 9 meters per min. The treadmill grade will be held at 0 degrees. The $\dot{V}O_2max$ protocol will consist of a starting speed of 10 meters per min for 3 min, and then increased by 0.05 meters per sec (3 meters per min). The treadmill grade will be held at 0 degrees. The test will be terminated when the mice are unable to maintain the running speed and are in contact with the electrical grid for 5 consecutive seconds. The modular system contains a fan that provides continuous flow during the running test. At the end of the $\dot{V}O_2max$ test, blood lactate will be measured from a small quantity of blood (< 0.010 ml) collected from the tail vein. Mice will be be exposed to air (n = 32) or HBO2 at 3 ATA (n = 32) for 60 min. Mice will be pre-treated (IP injection) 30 min before each air or HBO2 exposure with TGB (9.4 mg/kg, n = 16) or vehicle (0.9% NaCl, n = 16). Mice will complete a second $\dot{V}O_2max$ test after the exposure.
Category D: 64

Describe the anticipated sequence of experimental events (timeline) such as breeding, preparation of animals, surgery, testing procedures, collection of tissues, euthanasia, etc.:

(b) (4) [redacted]

Experiment 1: Mice will be brought to (b) (4) the day before the experiment.

Dive profile 1: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.

Dive profile 2: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be placed back in their cages housed in room (b) (6). (b) (4) [redacted] and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.

Dive profile 3: On experimental days, mice will receive an IP injection of TGB or vehicle, be exposed to HBO2 at 5 ATA in individual chambers for 60 min and monitored for signs of toxicity. After exposure to HBO2, they will be placed back in their cages (b) (6) [redacted]. (b) (4) [redacted] and monitored for signs of toxicity. After exposure to HBO2, they will be removed from the hyperbaric chamber and quickly euthanized by inhalation of isoflurane.

Experiment 2: Once the (b) (4) [redacted] are generated, bred, and transferred to GSRBII, they will be be brought to (b) (4) the day before the experiment. The mice will undergo the same dive profiles described in experiment 1. After the final HBO2 exposure, mice will be placed back in their cages housed in (b) (6) [redacted]. Mice will be quickly euthanized by inhalation of isoflurane 24- (n = 16, 48- (n = 16) and 72-hours (n = 16) later.

Experiment 3: Mice will be brought to (b) (4) the day before the experiment. Mice will be familiarized to the rodent treadmill and perform a baseline $\dot{V}O_2max$ test over one week. On a different day, mice will receive an IP injection of TGB or vehicle and be exposed to air or HBO2 at 3 ATA. After exposure, the mice will complete a second $\dot{V}O_2max$ test. Blood will be collected from the tail vein after $\dot{V}O_2max$ testing. After the second $\dot{V}O_2max$ test and blood collection, mice will be be quickly euthanized by inhalation of isoflurane.

B11. USE OF ANIMALS FOR PERSONNEL TRAINING

Is personnel training the primary purpose of this protocol?

- (●) No, this is not a training protocol. However, I may use small numbers of the approved experimental animals to train my research staff the procedures approved on this protocol.
- ( ) Yes, animals will be used for **non-medical personnel training** (students or others).
- ( ) Yes, animals will be used for **medical personnel training (MD, RN, OD, DC, DVM, etc.)**. Please review Survey Form for Medical Education Using Animals for instructions on completion of the participant survey, and a copy of the survey.
- ( ) Yes, animals will be used for **medical student training**. Please review Survey Form for Medical Education Using Animals for instructions on completion of the participant survey, and a copy of the survey.

For **medical personnel training** and **medical student training,** describe how the use of animals enhances the didactic and simulator components of the training exercise:

B12. GRADES OF CHEMICALS OR SUBSTANCES ADMINISITERED TO ANIMALS:

Note 1: USDA (Policy #3) and The Guide (8th Edition) requires the use of pharmaceutical-grade substances **(medications, diluents, and extenders)** whenever they are available, even in acute procedures. See the Duke Policy on Non-Pharmaceutical Grade Substance Use and the Guidelines for details.

Note 2: Non-pharmaceutical grade chemical compounds may be used in animals only after specific review and approval by the IACUC for reasons such as scientific necessity or non-availability of an acceptable veterinary or human pharmaceutical-grade product. Cost savings is not a justification for using non-pharmaceutical-grade compounds (exceptions for extraordinary costs of substances may be considered).

Note 3: The IACUC may consider the grade, purity, sterility, pH, pyrogenicity, osmolality, stability, site and route of administration, formulation, compatibility, and pharmacokinetics of the chemical or substance to be administered, as well as animal welfare and scientific issues relating to its use when determining whether to approve the use of non-pharmaceutical grade products. Inclusion of this information will assist the Committee's review of your request for use of non-pharmaceutical grade material in animals.

Will this protocol include the use of non-pharmaceutical grade substances, medications, test compounds, vehicles, diluents?

- [x] No. All chemicals and substances used in animals will be pharmaceutical grade.
- [ ] Yes. Some or all chemicals and substances used in animals will be non-pharmaceutical grade. Complete the table below:

	Non-pharmaceutical chemical or substance Required	Scientifically justify why each non-pharmaceutical grade chemical or substance is necessary	Describe the source, purity, formulation, storage, and method of sterilization prior to administering to animals. Supporting documents (e.g., data sheet) should be placed in the document container. (Note: 3)
Add Chem. / Substance			

B13. PHOTOGRAPHY/VIDEOS OF ANIMALS OR TISSUES:

Will images/video be taken of live animals for scientific purposes/publication?

- [ ] No. Proceed to Section B14.
- [x] Yes. I will adhere to the Duke IACUC policy regarding photography of animals. Complete Section B13.

Are animals (tissues) Duke-owned, Client-owned, or Free-ranging?

- [ ] Free-ranging. Note: No approvals are necessary for free-ranging animals.
- [ ] Client-owned. Note: The Client should provide approval to photograph the animals. No Duke approval required.
- [x] Duke-owned. Please complete the following:

Provide the name(s) of the photographer(s) and description of how images/videos will be secured/ protected from unauthorized use:

(b) (6) and (b) (6) may obtain video recordings of the behavior of conscious mice exposed to HBO2. (b) (6) will keep the video recordings under lock and key in her office (Rm. (b) (6), white zone, Duke Clinic Building) and be responsible for their security.

Provide a description and purpose of the photographs/videos, including species (if not all listed in the protocol), and whether the photos are whole body, histologic/fresh/fixed tissues, or radiologic (e.g., CT, PET, MRI):

The video records will allow the investigators to review the behavior of small groups of conscious mice exposed together to hyperbaric oxygen, in order to accurately determine the times onset of CNS toxicity (seizure latency) for individual animals.

B14: UNIQUE STRAINS REGISTRY (MouseBase)

- ☒ I have no unique strains (or substrains) of mice in this proposal.
- ☐ I have registered my unique strains (or substrains) of mice with the Duke MouseBase registry.

SECTION C: HOUSING & PROCEDURE ARRANGEMENTS

C1. DLAR-MANAGED, PI-MANAGED, OR VA-MANAGED HOUSING

NOTE: Confirm housing space availability with DLAR Operations Manager prior to placing an animal order!

Check all that apply:

- ☐ No animal housing will occur at Duke (also includes field studies, collaborating institutions, etc.).
- ☒ DLAR-Managed housing ***NOTE: Contact*** (b) (6) ***to coordinate space requirements prior to ordering animals.***
  - ☐ CCIF-Managed housing ***NOTE: Contact*** (b) (6) ***to coordinate space requirements prior to ordering animals.***
  - ☐ Non-human Primate housing ***NOTE: Contact*** (b) (6) ***to coordinate space requirements prior to ordering animals.***
- ☐ Duke Lemur Center ***NOTE: Contact*** (b) (6).
- ☐ VA-Managed housing (A Duke Protocol is not required, a Duke MOU is required)
  NOTE 1: Contact (b) (6) ***for VA housing arrangements.***
  NOTE 2: Transport between the VA and Duke requires DLAR approval.
  NOTE 3: Holding/housing of, or research with animals at the VAMC requires VA Animal Component of Research Protocol (ACORP).
- ☐ Duke PI-Managed housing ***NOTE: Enter the location on the table below and complete Section T.***

Species: **Mus musculus**

ACTIVITY		BUILDING	ROOM
PI Managed Housing/Holding **(or VA-Managed Housing/Holding)** (DO NOT complete this row if DLAR Housing)	Greater than 24 hours	(b) (4)	(b) (6)
	Less than 24 hours		
Survival Surgery	Pre-operative preparation		
	Surgery		
	Post-operative care		
Non-Survival Surgery	Pre-operative preparation		
	Surgery		
Nonsurgical Procedures (including euthanasia)		(b) (4)	(b) (6)

ACTIVITY	BUILDING	ROOM
Hazardous Agent Use (location of animals while excreting agent)		
Breeding Colony Activities		

Add Species

C2. SPECIAL HUSBANDRY REQUIREMENTS

Is approval requested for any special husbandry needs? Note that special husbandry needs that are approved must be implemented through direct arrangements with DLAR or Director of the relevant housing facility.

- (●) No. There are no special husbandry requirements.
- ( ) Yes. There are special husbandry needs.

C3. END-OF-STUDY HOUSING

While protocol planning which minimizes animals remaining as single animals for extended periods of time is preferred, on occasion, as experiments approach the end, a single animal may remain. Please answer the following:

- ( ) There will be no end-of-study animals maintained as single animals.
- (●) There may be end-of-study animals which will be maintained as single animals for a limited period of time. I confirm that I will provide an enhanced level of species-specific environmental enrichment for any end-of-study animal being singly housed. I understand that an IACUC Exemption for Single Housing is not required for end-of-study animal(s) housed singly for periods less than 30 days. I shall promptly notify the IACUC if the end-of-study single housing extends beyond 30 days.

SECTION D: SPECIAL CONCERNS FOR ANIMAL USE

D1. EXEMPTION FROM ANIMAL WELFARE STANDARDS

Are there experimental or scientific reasons why any animal on this protocol should be exempted from animal welfare standards?

- (●) No.
- ( ) Yes. An exemption from *Housing Density Standards* is requested in Section U.
- ( ) Yes. An exemption from *Social Housing Standards* is requested in Section U.
- ( ) Yes. An exemption from *Cage Change Frequency Standards* is requested in Section U.
- ( ) Yes. An exemption from *Environmental Conditions (temp, humidity, light, HVAC) Standards* is requested in Section U.
- ( ) Yes. An exemption from *Feed / Water Provision Standards* is requested in Section U.
- ( ) Yes. An exemption from *Radio / Sounds / Noise Standards* is requested in Section U.
- ( ) Yes. An exemption from *Environmental Enrichment Standards* is requested in Section U.
- ( ) Yes. An exemption from *Aquatics Water Quality Standards* is requested in Section U.
- ( ) Yes. An 'OTHER' exemption is requested in Section U.

Explain:

D2. ETHER *(NOTE: The use of ether presents serious animal and researcher safety concerns and requires compelling scientific justification.)*

Will ether be used for euthanasia, anesthesia, or brief sedation?

- (●) No. Ether will not be used.
- ( ) Yes. Ether must be used for scientific reasons.

D3. CONTROLLED SUBSTANCE USE

Will controlled substances be used for anesthesia, restraint, animal management, agent testing, or euthanasia?

- (●) No. Controlled substances will not be used.
- ( ) Yes. Controlled substances will be used, but I will not obtain a controlled substance registration. The DLAR/DLC Technical Services protocol will provide necessary controlled substances for sedation, anesthesia, analgesia, and euthanasia; and DLAR/DLC provision of controlled substances will continue for the duration of this protocol.
- ( ) Yes. Controlled substances will be used. I DO NOT have the registrations at present, but I am/will apply for controlled substance registration. Until my controlled substances registrations are approved by the state & federal agencies, I will use the DLAR/DLC Tech Services protocol for necessary controlled substances used for sedation, anesthesia, analgesia, and euthanasia. An Amendment will be submitted when the state and federal registration numbers are received to transfer authorized controlled substance use off of the DLAR/DLC Tech Services protocol and to my protocol
- ( ) Yes. Controlled substances will be used. A Unit Controlled Substance Registration or an Individual Controlled Substance Registration will provide controlled substances for sedation, anesthesia, analgesia, and euthanasia. DLAR/DLC veterinary staff may provide controlled substance administration at my request. My controlled substance registrations are:

* Registrant Name: (NOTE: The Registrant of the C.S. License / Registration MUST be listed in Protocol Section A-3 (Roles).

* N. Carolina DHHS #: _____ * Federal DEA #: _____

* Location of C.S. storage (bldg/rm):

D4. PHYSICAL RESTRAINT

Will the proposed research require the use of physical restraint (other than short-term hand-held) of awake animals?

- (●) No. Physical restraint of awake animals will not exceed short-term hand restraint.
- ( ) Yes. Physical restraint must be used. I recognize and agree with the following:

- [ ] Restraint devices shall not be considered a normal method of housing.
- [ ] Restraint devices shall not be used as convenience in handling or managing animals.
- [ ] Alternatives to physical restraint have been considered and cannot meet the requirements of my study.
- [ ] The restraint period shall be the minimum required to accomplish my research objectives.
- [ ] Animals shall be acclimated to the devices and personnel prior to actual research use.
- [ ] Animals that fail to adapt shall be removed from the study.
- [ ] Animals in restraint shall be observed at appropriate intervals, as described below.
- [ ] Veterinary care shall be provided if lesions or illnesses associated with restraint are observed.
- [ ] Clear explanation of the purpose of the restraint and its duration shall be provided to personnel involved.

Please address D4a - f below. If the species is a non-human primate, also complete D4g.

D4a. Briefly describe or identify the restraint device:

D4b. Briefly describe the procedure for restraining the animal:

D4c. State the duration of the restraint period:

D4d. Describe the plan for observation of the animal during the period of restraint:

D4e. Describe the plan for animal care and support during the period of restraint to ensure comfort and well-being:

D4f. Describe the procedure for conditioning the animal to the restrain device and procedure so as to minimize potential animal distress during restraint:

D4g. (Non-human primates only) Describe the plan for providing special attention to environmental enrichment during the period of restraint or restricted activity:

D5. WITHHOLDING OF ANESTHETICS OR ANALGESICS

Does this protocol involve procedures that are expected to cause pain, but for which pain-relieving anesthetics and/or analgesics will not be provided?

- ○ No. There are no painful procedures (i.e., no greater pain than would be expected from simple injections).
- ○ No. Anesthetics and/or analgesics will be provided for pain relief.
- ◉ Yes. This protocol includes painful procedures for which anesthetics and/or analgesics must be withheld. Please complete D5a and D5b below.

D5a. Identify the treatment groups and specify the number of animals in each group that will not be provided with pain relief.

Conscious mice breathing 5 ATA oxygen for 60 min (n = 432).

D5b. Detail the scientific reason that requires the withholding of anesthetics and/or analgesics for pain relief.

The purpose of this research is to examine how HBO2 and GABA influences seizures and oxidative tissue damage. The use of anesthetics or analgesics will delay seizure onset or modify neurotoxic behavior. In addition, our goal is to find prevention strategies that can be transitioned into human testing, requiring the use of conscious mice.

D6. ANIMAL WELL-BEING, HUMANE & EXPERIMENTAL ENDPOINTS

According to The Guide, researchers should consider the impact of their procedures upon the animals' well-being. Do you anticipate any animal health complications (e.g. local or systemic infection, physical or physiological impairment, heavy tumor burden, tumor necrosis, malnutrition, dehydration, etc.) arising from the experimental procedures or animal manipulations that are proposed in this protocol?

- ○ No. Animal health complications are not expected. Answer only D6d & D6e.
- ◉ Yes. Animal health complications may occur. Answer D6a through D6e.

D6a. Describe the health complications that are anticipated:

Seizures: Typically last between seconds to a few minutes. We have seen different stages from Stage 1, immobility; Stage 2, forelimb and/or tail extension, rigid posture; Stage 3, repetitive movements, head bobbing. If mice showed extended time of seizure animals will be removed and immediately euthanized. TGB is an FDA approved drug and safe for humans and mice with no reported toxicity.

D6b. Describe the plan for detecting the development of complications and their routine management:

Animals will be constantly monitored during HBO2 for signs of seizure.

D6c. If animals experience complications that are not resolved by the above management plan, specify the action(s) that will be taken (select all that apply).

- ☒ Euthanize the animal.
- ☐ Seek veterinary care from a Duke veterinarian.
- ☐ Withdraw the animal from the study for treatment and recovery according to the following plan (describe):

D6d. According to The Guide, the Principal Investigator, with precise knowledge of both the objectives of the study and the proposed model, should identify, explain and include in the protocol a study endpoint that is both humane and scientifically sound. The experimental endpoint of a study occurs when the scientific aims and objectives have been reached. The humane endpoint is the point at which pain or distress is prevented, terminated or relieved in an experimental animal. The use of humane endpoints contributes to refinement by providing an alternative to experimental endpoints that result in more severe animal pain and distress, including death. Please select the correct response.

- ☒ I anticipate that the experimental endpoints will be reached prior to the humane endpoints
- ☐ I anticipate that humane endpoints will be reached prior to the experimental endpoints. This is necessary because:

D6e. Other than euthanasia, is there the potential for adverse outcomes affecting the animal's well-being (inflammation, inappetence, etc.)?

☐ No, other than euthanasia, there is no potential for adverse outcomes affecting the animal's well-being.

☒ Yes, other than euthanasia, there is the potential for adverse outcomes affecting the animal's well-being. Explain:

HBO2 seizures.

D7. ACCLIMATION AND HABITUATION OF RESEARCH ANIMALS

According to The Guide, habituating animals to routine husbandry or experimental procedures may assist the animal to better cope with a captive environment by reducing stress associated with novel procedures or people. (See Policy on Acclimation) The type and duration of habituation needed will be determined by the complexity / novelty of the procedure. Please select the appropriate response:

Acclimation upon arrival to Duke:

☒ The Policy on Acclimation will be followed.

☐ I am unable to adhere to the Policy on Acclimation. My justification is:

Habituation for Husbandry or Experimental Procedures

☐ Habituation is not necessary. This protocol does not present the animal with novel procedures or people which would require habituation or training to assist the animal with coping.

☒ Habituation / training are performed as part of my research protocol. The methods I will use include:

For involuntary running exercise, mice will be placed on the treadmill set at a 0% incline. The treadmill speed will be increased from 0 to 3 meters per min for 2-6 min (depending on habituation session), increased to 6 meters per min for 2-5 min (depending on habituation session), and then increased to 9 meters per min for 2-4 min (depending on habituation session). Total habituation sessions will be 10-minutes. The shock grid will be set at 0.2 mA, 200 ms pulses, 1 Hz. If during the habituation sessions the mice touch the shock grid for 5 consecutive seconds, the speed will be reduced until the mice resume running.

SECTION E: EUTHANASIA & DISPOSITION

E1. EUTHANASIA:

Please indicate the role of euthanasia in the proposed activity:

☐ Euthanasia is not planned, but if necessary will be performed to prevent animal distress using one of these options:
1) A Duke veterinarian will perform or order to be performed an AVMA-approved method of euthanasia.
2) For situations (e.g. field studies) where no veterinarian is available, the method selected below will be used.

☐ Euthanasia will not be performed. Federal, international, or local permits governing this work do not allow euthanasia.

☒ Animals will be euthanized as part of the experimental protocol. (Specify method below.)

Please specify the method(s) of euthanasia below. Use a separate line for each species studied under this protocol.

Generally Acceptable Methods Of Euthanasia ***(See drop-down list for acceptable options)*:**

	Species	Method	Anesthetic or Injectable Agent / Dose / Route of Administration or Tricaine Concentration
Add Species	Mus musculus	Inhalant anesthetic gas (not ether)	Isoflurane in a bell jar on 4x4 gauze below a raised platform to separate mice for contact with the isoflurane.

NOTE: ***ALL PRIMARY METHODS OF EUTHANASIA require a secondary method*** to ensure non-recovery.
Please select the secondary method(s) that will be used to ensure euthanasia:

- ☒ Bilateral thoracotomy ☐ Decapitation
- ☒ Tissue / organ collection. Specify tissues / organs: _____
- ☒ Other method to ensure death Specify: cervical dislocation

Conditionally Acceptable Methods Of Euthanasia ***(See drop-down list for acceptable options)*:**

	Species	Method	Scientific Justification for Procedure without Anesthesia
Add Species			

Other Methods of Euthanasia ***(consistent with AVMA Panel on Euthanasia recommendations)***

	Species	Method (specify)	Agent / Dose / Route of Administration as Applicabale
Add Species			

Please explain the reason for performing euthanasia by a method other than those identified above as generally or conditionally acceptable:

E2. FINAL DISPOSITION OF ANIMALS

Live animals may be transferred to other approved Duke protocols to facilitate collaborative interactions and reduce overall animal usage and undue wastage. All transfers must be coordinated through DLAR (or DLC for lemurs), unless otherwise specified. In addition to the default method of disposition as described, please also indicate other method(s) of terminating responsibility for the live animals:

- ☐ Live animals may be returned to production / breeding unit.
- ☐ Live animals may be transferred to DLAR for disposition by DLAR as appropriate (requires prior approval by DLAR).
- ☒ Animals will be euthanized by methods specified in section E1 above (Euthanasia).
- ☐ Other (specify): _____

E3. FINAL DISPOSITION OF TISSUES, FLUIDS, OR CARCASSES

Indicate the method(s) of disposing of the carcasses and surplus tissues or fluids (select all that apply):

- ☒ Carcasses of non-biohazardous dead animals will be disposed of by DLAR.
- ☐ Non-radioactive tissues or fluids will be disposed of by the PI.
- ☐ Radioactive carcasses, tissues, or fluids will be disposed of by OESO.
- ☐ Other (specify): _____

E4. SHARING OF TISSUES, FLUIDS, OR CARCASSES

NOTE: This section ONLY APPLIES to animals which are dead prior to collecting the tissues, fluid, or carcasses. Any collection of tissues or fluids from animals which are alive requires specific protocol approval for the collection of tissues or fluids.

Indicate below if you anticipate sharing tissues, fluids, or carcasses post-euthanasia from this protocol:

- ☒ I may share tissues, fluids, or carcasses from my euthanatized animals with Duke researchers.
- ☐ I may share tissues, fluids, or carcasses from my euthanatized animals with non-Duke researchers. Note: Prior to shipping off campus, check with DLAR and OESO for requirements of biological specimen shipment.

SECTION F: PRINCIPAL INVESTIGATOR AGREEMENT

Check each box that is applicable. A checked box indicates agreement by the PI for the statement checked.

- ☒ I will conduct the project in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), and the *Guide for the Care and Use of Laboratory Animals*.
- ☒ I have determined that the research proposed is not unnecessarily duplicative.
- ☒ I confirm that all individuals working on this protocol have been assessed for health risks and are participating in an appropriate Occupational Health & Safety Program. (Note: The Duke Animal Handler Health Review forms are located at http://www.safety.duke.edu; participation in an Occupational Health Program is mandatory).
- ☒ I authorize individuals listed on this application to conduct procedures involving animals and I accept responsibility for their oversight in the conduct of this proposal.
- ☒ I confirm that all individuals listed on this protocol as working with animals have completed the online Animal Handler training or will be required to do so before being permitted to begin work with animals. Further, I certify that those individuals are properly trained, or will receive such training prior to working with animals, in all areas relevant to their assigned work with animals (e.g., biology, handling, and care of the species used; aseptic surgical methods and techniques; the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers; and procedures for reporting animal welfare concerns).
- ☒ For animals under this proposal, I understand that in cases of necessary medical treatment, Duke University veterinarians are authorized to provide any treatment required to sustain life; or if necessary, provide humane euthanasia to prevent unapproved distress and/or pain. I recognize that the veterinary staff will contact me as soon as possible using the emergency contact information that I provide in this application, but I understand that such contact may not always be possible prior to providing treatment or performing euthanasia.
- ☒ I will notify the IACUC (IACUC@duke.edu) of unanticipated outcomes of animal use, including protocol and non-protocol disease or injury. Unanticipated outcomes are generally defined as negatively impacts to animal welfare or well-being. This includes animals at Duke or animals funded by Duke but residing at collaborating institutions.
- ☒ I recognize that veterinary consultation must occur when pain or distress is beyond the level IACUC approved in this protocol, or when my staff are unable to provide interventional control (e.g., euthanasia, immediate removal from the study). For animals held or used on the Duke campus, I will notify a DLAR or DLC veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs.
- ☒ I will obtain approval from the IACUC before initiating any change in the study design or procedures by submitting a request for minor or significant change as appropriate. I understand that work performed without IACUC approval cannot be published with certification of IACUC approval and may result in federally-required reporting of non-compliance.
- ☒ For all USDA Category D (anesthesia / analgesia provided to relieve potential pain) and USDA Category E (pain not relieved by anesthesia / analgesia) animal use procedures, I certify that I have reviewed the pertinent scientific literature and the sources and/or databases noted in this application and found no scientifically acceptable alternative to any of those procedures that would result in less pain or distress.

		Animal Handling Role
PI Name: Heath Gasier	Duke Unique ID (b) (6)	◉ YES ○ NO
Sponsor Name (if applicable):	Duke Unique ID	○ YES ○ NO

Project Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

NOTE: Signature is not necessary when emailed from the PIs 'duke.edu' email address.
If this project has a sponsor, then the sponsor should be listed in the 'cc' box of the email.

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): Gasier, Heath Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b)(6) [redacted]

Dept / Div: Anesthesiology/GVT Campus Box #: (b) (6) Day Phone #: (b) (6)

Highest Degree Earned: ☐ High School ☐ AA / AS ☐ BA / BS ☐ MA / MS ☒ MD/ PhD/ DVM

☐ Other (specify):

Duke Relationship (Check all that apply): ☒ Faculty ☐ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke

☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☐ Permanent Staff ☐ Temporary Staff

○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	09/05	☐	☒	09/05	☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☒	09/05	☐	☒	09/05	☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☒	09/05	☐	☒	09/05	☐
Intraperitoneal	☒	09/05	☐	☒	09/05	☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	09/05	☐	☒	09/05	☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☒	09/05	☐	☒	09/05	☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☒	09/05	☐	☒	09/05	☐
Cutdowns	☒	09/05	☐	☒	09/05	☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒	09/15	☐	☒	09/15	☐
Aseptic technique	☒	09/05	☐	☒	09/05	☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	09/15	☐	☒	09/15	☐
Inhalation	☒	09/15	☐	☒	09/15	☐
Injectables	☒	09/05	☐	☒	09/05	☐
Decapitation	☒	09/05	☐	☒	09/05	☐
Cerv. dislocation	☒	09/05	☐	☒	09/05	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	09/05	☐	☒	09/05	☐
Decap.	☒	09/05	☐	☒	09/05	☐
Organ Removal	☒	09/05	☐	☒	09/05	☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

Animal Handling, Texas A&M University, 2005. Investigator Animal Use, Uniformed Services University of the Health Sciences, 2015.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

Sixteen years of experience with mice and rats. HBO2 monitoring, exercise training, non-survival surgeries, blood collection, injections, euthanasia, tissue collection.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)

Dept / Div: Anesthesiology-Hyperbaric Division Campus Box #: (b) (6) Day Phone #: (b) (6)

Highest Degree Earned: ☐ High School ☐ AA / AS ☐ BA / BS ☐ MA / MS ☒ MD/ PhD/ DVM ☐ Other (specify):

Duke Relationship (Check all that apply): ☒ Faculty ☐ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke ☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☐ Permanent Staff ☐ Temporary Staff

◉ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

○ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☐ Animal Handler Health Review clearance through EOHW- Annual requirement

- ☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates
- ☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

- ☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.
- ☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.
- ☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.
- ☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.
- ☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.
- ☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

- ☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.
- ☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).
- ☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.
- ☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.
- ☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.
- ☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.
- ☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.
- ☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☐		☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☐		☐	☐		☐
Intraperitoneal	☐		☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☐		☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☐		☐	☐		☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☐		☐	☐		☐
Inhalation	☐		☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☐		☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☐		☐	☐		☐
Decap.	☐		☐	☐		☐
Organ Removal	☐		☐	☐		☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)

Dept / Div: Anesthesiology-Basic Sciences Campus Box #: (b) (6) Day Phone #: (b) (6)

Highest Degree Earned: ☐ High School ☐ AA / AS ☒ BA / BS ☐ MA / MS ☐ MD/ PhD/ DVM ☐ Other (specify):

Duke Relationship (Check all that apply): ☐ Faculty ☐ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke ☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☒ Permanent Staff ☐ Temporary Staff

○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/13	☐	☒	07/01/13	☐
Oral Gavage	☒	07/01/13	☐	☒	07/01/13	☐
Injections						
Intravenous	☒	07/01/13	☐	☒	07/01/13	☐
Intramuscular	☒	07/01/13	☐	☒	07/01/13	☐
Subcutaneous	☒	07/01/13	☐	☒	07/01/13	☐
Intraperitoneal	☒	07/01/13	☐	☒	07/01/13	☐
Intracardiac	☒	07/01/13	☐	☒	07/01/13	☐
Blood collection						
Tail vein	☒	07/01/13	☐	☒	07/01/13	☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☒	07/01/13	☐	☒	07/01/13	☐
Cardiac puncture	☒	07/01/13	☐	☒	07/01/13	☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☒	07/01/13	☐	☒	07/01/13	☐
Neuroleptics	☒	07/01/13	☐	☒	07/01/13	☐
Volatiles	☒	07/01/13	☐	☒	07/01/13	☐
Aseptic technique	☒	07/01/13	☐	☒	07/01/13	☐
Post Surgical Care	☒	07/01/13	☐	☒	07/01/13	☐
Euthanasia						
CO_2	☒	07/01/13	☐	☒	07/01/13	☐
Inhalation	☒	07/01/13	☐	☒	07/01/13	☐
Injectables	☒	07/01/13	☐	☒	07/01/13	☐
Decapitation	☒	07/01/13	☐	☒	07/01/13	☐
Cerv. dislocation	☒	07/01/13	☐	☒	07/01/13	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/13	☐	☒	07/01/13	☐
Decap.	☒	07/01/13	☐	☒	07/01/13	☐
Organ Removal	☒	07/01/13	☐	☒	07/01/13	☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

Training by DLAR staff as required for RACC certification.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have been working with rodents for 20+ years and have experience injecting mice as well as euthanasia.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Treadmill running and HBO2 monitoring. Dr. Heath Gasier will provide the requisite training for these procedures.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6) [redacted] Duke Unique ID: (b) (6) [redacted] Date of This Form: Oct 6, 2021

Net ID: (b) (6) [redacted] E-mail Address (b) (6) [redacted]

Dept / Div: House Staff - Medicine Campus Box #: (b) (6) [redacted] Day Phone #: (b) (6) [redacted]

Highest Degree Earned: ☐ High School ☐ AA / AS ☐ BA / BS ☐ MA / MS ☒ MD/ PhD/ DVM

☐ Other (specify): _____

Duke Relationship (Check all that apply): ☐ Faculty ☒ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke

☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☐ Permanent Staff ☐ Temporary Staff

○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse			rat		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/20	☐	☒	07/20	☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☒	08/18	☐	☒	08/18	☐
Subcutaneous	☒	08/18	☐	☒	08/18	☐
Intraperitoneal	☒	09/20	☐	☒	08/18	☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☐		☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☒	07/20	☐	☒	07/20	☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☒	07/20	☐	☒	07/20	☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒	08/18	☐	☒	08/18	☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	07/20	☐	☒	07/20	☐
Inhalation	☒	07/20	☐	☒	07/20	☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☒	07/20	☐	☒	07/20	☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	09/20	☐	☒	07/20	☐
Decap.	☒	09/20	☐	☒	07/20	☐
Organ Removal	☒	09/20	☐	☒	07/20	☐

Add skills for additional species

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

(b) (6) [redacted], 2018: rat handling, anesthesia, intubation, ventilation, nonsterile surgical procedures, euthanasia, post-mortem dissection for organ harvesting. Also 2018 online training on rat and mice handling, anesthesia, injections.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

Prior work experience with rats at (b) (6) [redacted] has prepared me for handling rats, rat euthanasia, and post-mortem dissection for organ harvesting. Lab shadowing here at Duke has prepared me for the same procedures in mice.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)

Dept / Div: Anesthesiology Campus Box #: (b) (6) Day Phone #: (b) (6)

Highest Degree Earned: ☐ High School ☐ AA / AS ☒ BA / BS ☐ MA / MS ☐ MD/ PhD/ DVM ☐ Other (specify):

Duke Relationship (Check all that apply): ☐ Faculty ☐ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke ☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☒ Permanent Staff ☐ Temporary Staff

○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☒ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mice					
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/10	☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☐		☐	☐		☐
Intramuscular	☐		☐	☐		☐
Subcutaneous	☒	07/01/10	☐	☐		☐
Intraperitoneal	☒	07/01/10	☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	01/10/21	☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☐		☐	☐		☐
Cutdowns	☐		☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☒		☐	☐		☐
Aseptic technique	☒	01/10/21	☐	☐		☐
Post Surgical Care	☒	07/01/10	☐	☐		☐
Euthanasia						
CO_2	☒	07/01/10	☐	☐		☐
Inhalation	☒	07/01/10	☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☐		☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/10	☐	☐		☐
Decap.	☐		☐	☐		☐
Organ Removal	☒	07/01/10	☐	☐		☐

Add skills for additional species

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

I have received my training at the Center for Hyperbaric Medicine and Environmental Physiology over the previous 11 years.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have been working with mice for 10+ years and am skilled in euthanasia, blood and tissue collection.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Treadmill running and HBO2 monitoring. Dr. Heath Gasier will provide the requisite training for these procedures.

Add another PQF form

SECTION G: PERSONNEL QUALIFICATIONS FORM (PQF)

(Duplicate for each individual listed in Section A3)

G1. PERSONAL INFORMATION

Name (Last, First): (b) (6) Duke Unique ID: (b) (6) Date of This Form: Oct 6, 2021

Net ID: (b) (6) E-mail Address (b) (6)

Dept / Div: Medicine/Pulmonary Campus Box #: (b) (6) Day Phone #: (b) (6)

Highest Degree Earned: ☐ High School ☐ AA / AS ☒ BA / BS ☐ MA / MS ☐ MD/ PhD/ DVM ☐ Other (specify):

Duke Relationship (Check all that apply): ☐ Faculty ☐ Resident/Fellow ☐ Visiting Scientist ☐ Not Paid by Duke ☐ Student - High S. ☐ Student - Undergrad. ☐ Student - Graduate ☒ Permanent Staff ☐ Temporary Staff

○ I **WILL NOT HAVE** animal contact. (Complete sections G1 and G2 of this form)

◉ I **WILL HAVE** animal contact. (Complete sections G1, G2, and G3 of this form)

Medical Health Surveillance Status *(For personnel with animal contact only)*

☒ Animal Handler Health Review clearance through EOHW- Annual requirement

☐ PPD Test/Clearance- Annual requirement for personnel handling or sharing air space with non-human primates

☐ Measles Documentation-One time requirement for personnel handling or sharing air space with non-human primates

Training Completed: Please check those trainings which have been completed (available on LMS website): For instructions on how to register for the course click here.

☒ Animal Handlers, Regulations Impacting Animal Care and Use and Veterinary Care: Required once for all protocol participants <Section A-3>.

☒ Animal Handlers Annual Refresher Training: Initial requirement for all protocol participants <Section A-3> then annually each January.

☐ Controlled substances: Required every 3 years for all protocol participants <Section A-3> when controlled substances are in the protocol.

☒ CO_2 Euthanasia of Rodents: Required once for all protocol participants <Section A-3> when CO_2 is used for euthanasia.

☒ Hazardous Awareness for Animal Facilities: Required once for all animal handlers <Section A-3>.

☐ Working Safely with Nonhuman Primates: Required every 3 years of all protocol participants <Section A-3> working with live primates.

G2. PERSONNEL AGREEMENT

Check each box that is applicable to confirm agreement. The Duke Animal Program considers submission from a '@duke.edu'e-mail account as a valid signature.

☒ I will perform my duties in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals*, and other applicable regulations as appropriate for the species and activities I am doing.

☒ I have enrolled in an Occupational Health & Safety Program for animal handlers. (Note: Click here to select the Animal Handler Review forms for enrollment through the Duke Office of Employee Health & Wellness).

☒ I have reviewed, or will review, the protocol section under which I will be performing work. I accept responsibility for conducting my work in accord with the approved protocol.

☒ I will confirm that IACUC approval has been received before initiating any changes in the study design or procedures.

☒ I will promptly notify the P.I. regarding any unexpected study results that negatively impact the welfare of the animals.

☒ I will notify a DLAR veterinarian when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals.

☒ I understand that work performed without IACUC approval may result in federally-required reporting of non-compliance.

☒ I certify that I have reviewed the humane endpoints in this proposal and are competent to assess animal's well-being.

G3. SKILLS AND EXPERIENCE

Please indicate species and skills in which you are ALREADY PROFICIENT and skills in which you will receive training. To list skills for more than two species, the table can be replicated.

Techniques	Species			Species		
	mouse					
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Handling & Restraint	☒	07/01/11	☐	☐		☐
Oral Gavage	☐		☐	☐		☐
Injections						
Intravenous	☒	12/05/11	☐	☐		☐
Intramuscular	☒	07/01/05	☐	☐		☐
Subcutaneous	☒	12/05/11	☐	☐		☐
Intraperitoneal	☒	12/05/11	☐	☐		☐
Intracardiac	☐		☐	☐		☐
Blood collection						
Tail vein	☒	12/05/11	☐	☐		☐
Jugular vein	☐		☐	☐		☐
Leg veins	☐		☐	☐		☐
Maxillary vein	☐		☐	☐		☐
Saphenous vein	☐		☐	☐		☐
Ant. vena cava	☐		☐	☐		☐
Orbital sinus	☐		☐	☐		☐
Cardiac puncture	☒	07/01/05	☐	☐		☐
Cutdowns	☒	07/01/05	☐	☐		☐

Techniques	Species			Species		
NOTE: If the actual training month/day is unknown, the default will be '07/01' with the year of the training indicated.	Current Skills	Most Recent Training	Training Needed	Current Skills	Most Recent Training	Training Needed
Anesthesia						
Barbiturates	☐		☐	☐		☐
Neuroleptics	☐		☐	☐		☐
Volatiles	☐		☐	☐		☐
Aseptic technique	☐		☐	☐		☐
Post Surgical Care	☐		☐	☐		☐
Euthanasia						
CO_2	☒	07/05/16	☐	☐		☐
Inhalation	☒	12/05/11	☐	☐		☐
Injectables	☐		☐	☐		☐
Decapitation	☐		☐	☐		☐
Cerv. dislocation	☒	12/05/11	☐	☐		☐
	☐		☐	☐		☐
Secondary Method						
Bilat. Thoracot.	☒	07/01/10	☐	☐		☐
Decap.	☒	12/05/11	☐	☐		☐
Organ Removal	☐		☐	☐		☐
Add skills for additional species						

Please describe any formal training that you have received related to the use of laboratory animals. Include the institution, approximate dates, and a brief description of the training content:

I received refresher training on handling and restraint, injections, tail vein blood collection, and CO2 euthanasia (with the secondary methods of cervical dislocation, bilateral thoracotomy, and decapitation for pups) on 12/5/11 with (b) (6) [redacted] and (b) (6) [redacted] staff. I am also a Duke Certified Research Animal Coordinator.

What experience do you have that prepares you to perform the procedures and use the techniques required of you in this protocol? (Please be specific.)

I have over 25 years of experience working with mice at Duke and the Durham VAMC. I have performed all of the procedures in this protocol many times and I am good about keeping up with required refresher training.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION I: TRANSPORTATION

I1. NON-DLAR ANIMAL TRANSPORTATION ON CAMPUS

Will animal be transported between buildings on the Duke campus by other than DLAR vehicles and/or personnel?

- ○ No. DLAR will provide all animal transportation that is required.
- ◉ Yes. Animals will be transported on campus by protocol personnel. Please address the following points:

How many times will any given animal be transported between buildings on campus?

- ◉ One time only.
- ○ More than once.

Describe the type of caging that will be used for transport of animals between locations.

All animals will be transported in DLAR provided caging and/or paperboard container with paperboard lid. Standard mouse box with wire top and filtered cage top.

Indicate the practices and materials that will be used to protect the animals during transit (select all that apply).

- ☒ Cages will be carried by hand.
- ☒ Cages will be moved on a cart or rollable platform.
- ☒ Cages will be moved in a private vehicle (IACUC approval will be required).
- ☒ Cage tops or doors will be secured with a bungee cord or elastic band (no tape)
- ☒ Cages will be covered to prevent visualization of animals during transit.
- ☒ Cages will be moved before or after normal work hours.
- ☐ Other (please describe):

Will the animals be transported by private (non-DLAR) vehicle?

- ○ No.
- ◉ Yes. Please complete the following:

Model of vehicle	(b) (6) [redacted]
Make (brand) of vehicle	(b) (6) [redacted]
Tag Number of vehicle	(b) (6) [redacted] (b) (6) [redacted]
Location in vehicle where animals will be held (e.g. seat, floor, trunk)	Trunk floor

I2. NON-DLAR ANIMAL TRANSPORTATION OFF CAMPUS

Other than shipment from a commercial vendor, will animals be transported off campus at any time in a non-DLAR vehicle?
Note: Transportation in a private, non-DLAR vehicle requires prior IACUC inspection and approval of the vehicle.

- ◉ No. DLAR will provide all animal transportation that is required.
- ○ Yes. Animals will be transported off campus in a non-DLAR vehicle.

I3. ANIMAL TRANSPORTATION THROUGH PATIENT CARE AREAS

Will animals be transported into or through human patient care areas? (e.g. Duke North, Duke South Clinical Areas, Children's Hospital, etc.)

- ◉ No. Animals will not enter human patient care areas at any time.
- ○ Yes. Section O must be completed. An on-site review of the proposed transport path will be conducted by a Medical Center committee. Contact the Office of Animal Welfare Assurance for additional information.

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)

SECTION M: NON-SURGICAL PROCEDURES

NOTE: Repeat items M1 through M13 *for each species* that will have non-surgical procedures.

The following items M1 - M13 apply to *(identify species)*: Mus musculus

M1. NARRATIVE OF NON-SURGICAL PROCEDURES

Species: Mus musculus

Description of non-surgical procedures:

HYPERBARIC OXYGEN (HBO2) HBOEXPOSURES
After administration of tiagabine, mice will be exposed to elevated atmospheric pressures in a hyperbaric chamber for 60 min. Exposures will be up to 5 ATA. Mice will be placed in individual Plexiglass chambers that receive a continuous supply of 100% oxygen (flow through system). Food and water will not be provided during the exposures. The dive time in the chamber is 60 min of bottom time. There will be travel time to and from the target depth along with a small amount of time on the surface prior to and after the dive when the mice will be in the chambers. The maximum time without food/water will be no longer than 1.5-2.0 hours. The onset of seizures is usually accompanied by unconsciousness, however, because of the possibility that some of these animals may experience distress, they will be designated in USDA pain category E. Mice will be communally housed all other times.

INJECTIONS
Mice will be given a single IP injection of tiagabine or 0.9% NaCl. The injections will be performed with sterile needles (1/2 in 27g) and syringes in the lower abdomen of the mouse.

INVOLUNTARY RUNNING
Mice will be habituated to running on a rodent treadmill and complete a $\dot{V}O_2$max test before HBO2 experiments. Each mouse will be placed on the treadmill the treadmill set at a 0% incline. The treadmill speed will be increased from 0 to 3 meters per min for 2-6 min (depending on habituation session), increased to 6 meters per min for 2-5 min (depending on habituation session), and then increased to 9 meters per min for 2-4 min (depending on habituation session). Total habituation sessions will be 10-minutes. The shock grid will be set at 0.2 mA, 200 ms pulses, 1 Hz. If during the habituation sessions the mice touch the shock grid for 5 consecutive seconds, the speed will be reduced until the mice resume running. On a different day, mice will perform a $\dot{V}O_2$max test. Mice will be placed on the treadmill and the starting speed will be 3 meters per min. Speed will be increased by 0.05 meters per sec (3 meters per min). The treadmill grade will be held at 0 degrees. The test will be terminated when the mice are unable to maintain the running speed and are in contact with the electrical grid for 5 consecutive seconds. On a different day and after HBO2 exposures, mice will repeat the $\dot{V}O_2$max test in identical fashion as the first.

BLOOD LACTATE
After each $\dot{V}O_2$max test, a small volume of blood (< 0.010 ml) will be collect from the tail vein onto lactate test strips inserted into a handheld lactate analyzer. Only 0.003 ml is needed with this analyzer. A repeated measurement may be required if the initial value is outside of the expected lactate concentration.

M2. PRE-PROCEDURE ANIMAL SUPPORT (NOT ANESTHESIA)

Will special pre-procedural care be provided?

○ No. The procedures do not require special pre-procedural care.

◉ Yes. Specify pre-procedural actions that will be taken to prepare animals for the procedure(s) *(select all that apply)*:

- ☐ Physical exam
- ☐ Overnight food withdrawal
- ☐ Body temperature support
- ☐ Clipping of fur
- ☐ CBC (define blood sampling method: _____
- ☐ Chemistry profile (define blood sampling method): _____
- ☐ Ophthalmic ointment to eyes
- ☐ Iodine (or Chlorhexidine) + alcohol skin scrub, 3 alternating cycles
- ☒ Drugs (other than anesthetics and sedatives) or fluids (list agents below):

	Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent	Tiagabine (TGB)	9.4 mg/kg	IP	Once	Single dose, multiple days

M3. PRE-PROCEDURE ANESTHESIA / SEDATION / TRANQUILIZATION

Will pre-procedure anesthesia, sedation or tranquilization be provided to the animals?

- ◉ No. Drugs will not be administered to the animals prior to the procedure(s).
- ○ Yes. Pre-procedure drugs will be used to calm the animals. *(List below)*

	Drug	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Drug					

M4. INTRA-PROCEDURE ANIMAL SUPPORT (NOT ANESTHESIA)

Will special intra-procedure care be provided?

- ◉ No. The procedures do not require special intra-procedural care.
- ○ Yes. Specify intra-procedure care that will be provided to animals during the procedure(s) *(select all that apply)*:

☐ Intravenous fluids

☐ Ophthalmic ointment to eyes

☐ Other (specify): _____

☐ Heat to prevent hypothermia

☐ Cooling to prevent hyperthermia

☐ Other (specify): _____

☐ Drugs (other than anesthetics and sedatives) or fluids (list agents below):

	Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent					

M5. INTRA-PROCEDURE ANESTHESIA OR CHEMICAL RESTRAINT

Will intra-procedure anesthesia or chemical restraint be provided?

- ◉ No. The procedures do not require intra-procedural anesthesia or chemical restraint.
- ○ Yes. The procedure requires chemical restraint as described in Section J or anesthesia as described below:

	Anesthetic Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Agent					

M6. NEUROMUSCULAR BLOCKING AGENTS (PARALYTICS)

Will neuromuscular blocking agents (paralytics) be used at any time during the procedure?

- ◉ No. Neuromuscular blocking agents will not be used for the procedure.
- ○ Yes. Neuromuscular blocking agents will be used. *(Provide details below)*

	Paralytic Agent	Dose	Route of Administration	Frequency of Administration	Duration of Treatment	Reversal Agent (if appropriate)
Add Agent						

Please state why the use of paralytic agents during the procedure is necessary.

Please describe how adequate anesthesia will be ensured during the time the animal is undergoing neuromuscular blockade:

M7. MONITORING DEPTH OF ANESTHESIA DURING PROCEDURES

Indicate below the indices that will be used for monitoring animal condition and depth of anesthesia.

- ☐ Respiratory rate / effort
- ☐ Mucous membrane color
- ☐ Body temperature
- ☐ Oxygen saturation
- ☐ Heart rate
- ☐ Blood pressure
- ☐ Capillary refill time
- ☐ EKG
- ☐ Reflex (specify): _____
- ☐ Other (specify): _____
- ☐ Other (specify): _____

Specify the frequency at which the above indices will be recorded: _____

M8. POST-PROCEDURE ANIMAL SUPPORT

Will special post-procedure care be provided?

◉ No. The procedures do not require special post-procedural care.

○ Yes. Specify post-procedure care that will be provided to animals after the procedure(s) *(select all that apply)*:

- ☐ Heat to prevent hypothermia
- ☐ Cooling to prevent hyperthermia
- ☐ Other (specify): _____
- ☐ Intravenous fluids
- ☐ Ophthalmic ointment to eyes
- ☐ Other (specify): _____

M9. MONITORING DURING RECOVERY FROM ANESTHESIA *(if used)*

Indicate below the indices that will be used for post-procedure monitoring of animal condition during recovery from anesthesia (i.e., until sternal recumbancy is regained and maintained):

- ☐ Respiratory rate
- ☐ Mucous membrane color
- ☐ Body temperature
- ☐ Oxygen saturation
- ☐ Heart rate
- ☐ Blood pressure
- ☐ Capillary Refill Time
- ☐ EKG
- ☐ Reflex (specify): _____
- ☐ Other (specify): _____
- ☐ Other (specify): _____

Specify the frequency at which the above indices will be recorded: _____

M10. PAIN MANAGEMENT INTRA- OR POST-PROCEDURE

NOTE: The Duke IACUC encourages the use of pre-emptive analgesia for pain management. Analgesia should be provided as early as possible in the procedure if it is expected to be painful or result in residual pain, ideally before the procedure begins.

Is the procedure expected to cause pain or result in residual pain?

◉ No. The procedure is not expected to cause pain.

○ Yes. Pain during and/or after the procedure is likely.

If pain is expected, will analgesia be provided for pain relief?

○ No. Analgesia will not be provided. (justify the omission of analgesia below)

○ Yes. Analgesia will be provided. (specify details below)

If pain is expected and analgesia will not be provided, please explain why pain relief will be withheld:

If analgesia will be provided for pain relief, please list analgesics and dosing regimens below:

	Analgesic	Timing of Administration	Dose	Route of Administration	Frequency of Administration	Duration of Treatment
Add Analgesic						

M11. SPECIMEN COLLECTION FROM LIVE ANIMALS

Will specimens be collected from living animals during or after the procedure(s)?

○ No. Specimens will not be collected from living animals.

◉ Yes. Define the specimen type and collection details below.

☒ Fluids (e.g., blood, lymph, ascites, CSF, GI fluids, etc.

Fluid type (specify): blood Collection method: tail vein

Volume (mls) per collection: 0.003 Frequency of collection: 2

Method of disposal: Biohazards

☐ Solid Tissues

Tissue type (specify): _____ Collection method: _____

Volume (mm3) per collection: _____ Frequency of collection: _____

Method of disposal: _____

M12. HUMANE ENDPOINTS THAT WILL PROMPT INTERVENTION TO PREVENT CONTINUED PAIN OR DISTRESS

According to The Guide, information that is critical to the IACUC's assessment of appropriate endpoint consideration within a protocol includes precisely defining the humane endpoint (including assessment criteria); the frequency of animal observation; training of personnel responsible for assessment and recognition of the humane endpoint; and the response required upon reaching the humane endpoint. The IACUC has determined that the list below defines the commonly accepted clinical milestones which should be regarded as humane endpoints for most terrestrial animal studies . Choose all of those which are appropriate for the species being used. For each milestone, indicate the action that will be taken. Add other milestones (in the row marked 'other') if applicable for defining the humane endpoints for the proposed study.

Clinical Observation / Milestone	Applicable to my proposal	Frequency of observation	Protocol personnel will perform each of these observations	Response required upon reaching the humane endpoint.	Provide duration (days. weeks. etc.) of monitoring OR a scientific justification for not using the milestones listed
Infection unrelated to the protocol.	No				
Not eating or drinking (will require individual housing to effectively assess)	No				
Decreased fecal and urine output (will require individual housing to effectively assess)	No				
Delayed wound healing (requires checking at least daily until suture removal)	No				
Sudden behavioral change (Ex: aggression, guarding, hiding)	No				
Licking, biting, scratching of the operative / injection site (requires checking at least daily until suture removal)	No				
Poor posture or ambulating difficulty (Ex: tense, tucked-up, stiff gait)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Lost hair coat condition (Ex: ruffled fur, lack of grooming, piloerection)	No				
Sudden activity level change (Ex: restlessness, pacing, reluctance to move)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Unexpected sweating or salivation (Ex: stressed rodents salivate excessively when stressed)	No				
'Painful' facial expression (Ex: grimace, eyes dull, pupils dilated, pinning of ears)	No				
Oculonasal discharge (Ex: rats shed porphyrin pigment when stressed)	No				

Clinical Observation / Milestone	Applicable to my proposal	Frequency of observation	Protocol personnel will perform each of these observations	Response required upon reaching the humane endpoint.	Provide duration (days. weeks. etc.) of monitoring OR a scientific justification for not using the milestones listed
Teeth grinding (Ex: More common sign in rabbits, livestock)	No				
Signs of moderate to severe pain or distress that was not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Body weight loss exceeding 15% of free-feeding body weight relative to an age-matched reference. (Ex: Requires regular <q 48 hours> weighing)					
Self mutilation (requires checking at least daily until suture removal)					
Neurological disorders (e.g., seizures, blindness, ataxia) that were not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Cardiopulmonary disorders (e.g. sudden weakness, vascular collapse, coma) that were not anticipated by the study plan.	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Abnormal feeding or defecation for 48 hours (e.g., decreased feed or water intake and/or decreased fecal production that is unrelated to the study plan).	No				
Non-weight bearing for 72 hours (e.g., difficulty wa king, inability to maintain upright posture)	Yes	q 24 hrs	Yes	Euthanize	3 days post HBO2
Other humane endpoints which will be employed in this project:					
Other humane endpoints which will be employed in this project:					

Add Sections M1 through M13 for another species.

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)

SECTION P: GENETICALLY ENGINEERED (Tg/KO/etc) ANIMAL USE ONLY; NO BREEDING (If genetically engineered animals will be bred at any time during the 3-year life of this protocol, DO NOT complete this section; complete Section Q instead.)

P1. SOURCE OF GENETICALY ENGINEERED ANIMALS

Source of genetically-altered animals:

- ☒ Colony managed by a Duke investigator. Specify source protocol #: (b) (4) [redacted] _____
- ☐ Obtained from another institution (or non-Duke investigator). Identify source: _____
- ☐ Purchased from a vendor (e.g. Harlan, Charles River, etc.)

P2. SPECIES AND GENOTYPE

List species to be used, identify genetic lines, and indicate any induction method that is necessary

	Species	Strain / Genotype	Phenotype Induction
Add Species	Mus musculus	(b) (4) [redacted]	☐ Pharmacologically activated ☐ siRNA ☐ Environmentally activated ☐ Viral induced ☒ Constitutive (gene is always `on') ☐ Other *(specify)*: _____

P3. GENOTYPING

Will genotyping be performed under this protocol?

- ◉ No. Genotyping is not necessary for this protocol.
- ○ Yes. Genotyping will be performed on tissue obtained by the method(s) defined below.

☐ Tail snipping (mice). *Please review the* IACUC policy *for this procedure*.

- ○ The tail snip will be limited to a maximum of 5 mm of the tail tip.
- ○ The tail snip will exceed 5 mm of the tail tip.

  Specify the maximum length of tail tip that will be taken and explain the need for the larger tissue specimen:

- ○ The tail snip will be taken after 21 days of age.
- ○ The tail snip will be taken prior to 21 days of age.

  Justify the delayed genotyping and describe the anesthetic regimen to be used for the procedure:

☐ Oral swabs.

☐ Blood collection.

☐ Other *(describe)*:

P4. PHENOTYPIC EXPRESSION AND CARE PROVISIONS

Briefly describe the genetic alteration of interest:

The use of fluorescent proteins targeting mitochondria allow for selective measurement of mitophagy and architecture. (b) (4) [redacted]

(b) (4) [redacted]

The phenotypic expression of this genetic alteration will likely result in:

- ☒ No morphologic or functional impairment
- ☐ Increased early mortality
- ☐ Physical impairment as follows:
- ☐ Functional deficit as follows:
- ☐ Possible impairments that are presently unknown.

Please describe the monitoring plan that will be used for early detection of potential impairments:

Indicate unique or special animal care needs (e.g., feed, water, temperature, humidly, light, air flow, etc.) that will be required for these genetically-altered animals:

- ☒ No special care is required.
- ☐ Special care needs will be determined by the monitoring plan for detecting potential impairments.
- ☐ Special care must be provided for these animals as follows:

P5. RECORD KEEPING

Indicate the record-keeping system that will be used to document health surveillance and the maintenance of well-being for the genetically-engineered animals.

- ☒ Special care is not required and documentation will be provided by standard DLAR observation records.
- ☐ Special care outlined above will be documented by the record-keeping sheet attached to this application.
- ☐ Special care outlined above will be documented as follows:

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION T: PI MANAGED ANIMAL HOLDING FACILITY

Note: Please refer to the Policy on **PI Managed Housing Facilities** for definitions, approval processes, and other requirements.

T1. REQUEST TO HOLD ANIMALS IN A LABORATORY OR OTHER PI-MANAGED AREA

○ This request is for initial approval of a PI-managed Animal Holding Facility.

◉ This request for renewed approval of an existing PI-managed Animal Holding Facility.

Specify the location of the Animal Holding Facility: Building: (b) (6)

Room(s): (b) (6)

Will any of these animals have ABSL-2 or 3 agents involved? ◉ No ○ Yes

Indicate the type of animal holding or housing for that location:

○ Continual (expectation that one or more animals will be present at any given time).

◉ Frequent (one or more animals often present, but with periods of no animal presence).

○ Sporadic (one or more animals infrequently present,.e.g., 3-4 days/month, with long periods of no animals presence)

Animal species to be held (at each location if multiple rooms are specified):

Mus musculus

Maximum number of cages of animals at this location at any given time: 12 cages

Maximum number of animals at this location at any given time: 48

Maximum duration that any given animal will be held at this location: <4 weeks

Person responsible for the care of animals at this location *(name)*: (b) (6)

According to the NRC 8th Edition of The Guide, testing equipment should be designed in such a way as to allow surface disinfection between studies. Select the correction option:

☒ Yes, testing equipment is designed to allow disinfection between studies

☐ No, testing equipment is not designed to allow disinfection between studies. Please clarify:

T2. JUSTIFICATION FOR ANIMAL HOLDING FACILITY

Please explain the scientific justification for holding animals outside of standard housing facilities and state the reason(s) why standard housing would interfere with achieving the scientific objective(s):

1. Mice will be exposed to HBO2 on more than one occasion separated by 2-3 days, and euthanized up to 1 week after the final HBO exposure.

In addition to the scientific justification provided above, you may include below any other information that you believe important for the IACUC to consider in reviewing this request (optional response).

This study is focused on understanding the effects of repeated HBO2 exposure on the brain and lung, and if the dose of tiagabine used impacts running performance after HBO2. For these reasons, we need to house the animals outside of DLAR.

T3. REQUIRED CLEARANCES

If scientific need is established, the clearances listed below will be required before the IACUC will approve the holding of animals in a PI-managed facility. Either enter the date clearance was obtained or enter "pending" in the date column and e-mail or fax the specific clearance statement to the IACUC when it is obtained.

Clearance	Date Completed	Notes
HVAC assessment (room air flow, pressure)	2/3/2021	**NOTE: These assessments must be done annually**. **For Medical Center buildings**: Contact E&O at 919.684.3232 **For University buildings**: Contact FMD at 919.660.4230
OESO safety audit	2/22/2021	Contact OESO at 919.684.8822 to have a safety audit.
PI-Managed Husbandry SOP	11/5/2020	Submit Husbandry SOP to (b) (6) [redacted]
Complete the appropriate on-line Module: **-PI Managed Housing Training** **NOTE: Modules are the LMS Training site: (https://vmw-lmsweb.duhs.duke.edu/SabaLogin)**	3/16/2018	Notify (b) (6) [redacted] of completion of module Note: Module completion required for IACUC Inspection of the proposed housing/holding site.
Adverse Event Plan	8/1/14	Submit Adverse Event Plan to (b) (6) [redacted]
IACUC Subcommittee Inspection	10/11/21	Contact (b) (6) [redacted] to schedule an IACUC Subcommittee inspection.

**Completed in OAWA Office*

T4. AUTHORIZATIONS PAGE FOR THIS FACILITY'S ADVERSE EVENT PREVENTION, RECOGNITION, & MITIGATION PLAN

Names listed on this section confirms that the individual has reviewed the facility disaster plan and has agreed to implement when determined necessary.

	Facility Contact (Primary contact on line one, secondary contact on line two, etc.)	Duke Unique ID	Emergency Contact Number (generally a cell phone or pager)
Add Name	(b) (6) [redacted]	(b) (6) [redacted]	(b) (6) [redacted]

Duke IACUC

From: Dr Heath Gasier, Ph.D.
Sent: Wednesday, October 27, 2021 1:43 PM
To: Duke IACUC
Subject: RE: Renewal_A023-19-01
Attachments: A-G_Renewal for A023-19-01.pdf; I-Transportation_Renewal for A023-19-01.pdf; M-Non-Surgical_Procedures_Renewal for A023-19-01.pdf; P-Transgenic_or_Knockout_Animal_Use_Renewal for A023-19-01.pdf; T-PI Managed Housing_Renewal for A023-19-01.pdf

(b) (6),

Made the changes, as well as a couple other ones, so please use these documents going forward.

1. (b) (6) will have no handler role (changed).
2. (b) (6) will not be breeding animals (changed).
3. Also changed number of animals used on this protocol (+77 from what is listed; changed).
4. Section T added, will be housed in (b) (6) for <4 weeks.

Heath

From: Duke IACUC <iacuc1@win.duke.edu>
Sent: Wednesday, October 27, 2021 11:41 AM
To: Dr Heath Gasier, Ph.D. <heath.gasier@duke.edu>
Subject: RE: Renewal_A023-19-01

Good morning Dr. Gasier,

Thank you for your IACUC renewal application. I have reviewed it administratively and have the following clarifications:

- Section A3:
  - (b) (6) is indicated as having an animal handler role, however the role described and his Section G indicate a non-animal handler. Please confirm if he will have an animal handling role or not.
  - (b) (6) animal handling role is described as breeding, however there was no section Q attached for animal breeding. If this is needed please submit.
- Section A8 & C1:
  - You've checked the box for the supplemental section T for PI managed housing as well as listed Building (b) (6) as PI managed housing greater than 24 hours.
    - If you will be housing mice in a non-centrally managed location for greater than 24 hours, a Section T is required.
    - Please clarify if you will need PI managed housing or if this location will only be used for non-surgical procedures for less than 24 hours.
    - Reference: PI Managed Housing Facilities policy

Thank you,
(b) (6)
Office of Animal Welfare Assurance

(b) (6) [redacted]
Durham, NC 27705
Office (b) (6) [redacted]
https://dacup.duke.edu

From: Dr Heath Gasier, Ph.D. <heath.gasier@duke.edu>
Sent: Tuesday, October 26, 2021 3:53 PM
To: Duke IACUC <iacuc1@win.duke.edu>
Subject: Renewal_A023-19-01

This submission is for the upcoming renewal (1/22) for A023-19-01, PI Heath G. Gasier, (b) (6) [redacted].

vr

Heath

Heath G. Gasier, PhD, RD
Assistant Professor in Anesthesiology
Center for Hyperbaric Medicine & Environmental Physiology
(b) (6) [redacted]

(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Wednesday, October 27, 2021 2:12 PM
To: OESO Animal Protocols
Subject: 7N Gasier A224-21-11
Attachments: 7N Gasier A224-21-11_FCR.pdf

Good afternoon,

Please see the attached new protocol for your review.

Thank you,

(b) (6) [redacted]
Durham, NC 27705
(b) (6) [redacted]
https://dacup.duke.edu

(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Wednesday, October 27, 2021 2:15 PM
To: (b) (6) [redacted]
Subject: 7N Gasier A224-21-11 - Vehicle and PI-Managed Housing
Attachments: 7N Gasier A224-21-11_FCR.pdf

Good afternoon,

Please see the attached new protocol that has a vehicle and PI Managed housing for facilities clearance.

Thank you,

(b) (6) [redacted]
Durham, NC 27705
(b) (6) [redacted]
https://dacup.duke.edu

(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Wednesday, October 27, 2021 2:50 PM
To: (b) (6) [redacted]
Cc: Dr Heath Gasier, Ph.D.; IACUC@DUKE.EDU
Subject: RE: Facility Review of New Protocol Submission: A224-21-11

Thank you, (b) (6) [redacted]

This resolves my concern. Protocol A224-21-11 is now cleared for facilities.

(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Wednesday, October 27, 2021 2:45 PM
To: (b) (6) [redacted]
Subject: RE: Facility Review of New Protocol Submission: A224-21-11

(b) (6) [redacted]

That is correct.

Thanks,
(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Wednesday, October 27, 2021 2:43 PM
To: Dr Heath Gasier, Ph.D. <heath.gasier@duke.edu>
Cc: (b) (6) [redacted] Duke IACUC <iacuc1@win.duke.edu>
Subject: Facility Review of New Protocol Submission: A224-21-11

Good afternoon Dr. Gasier,

I am performing a facility review of your recently submitted protocol and have just one clarification:

- Section I includes the (b) (4) [redacted] Ford (b) (6) [redacted]. In my latest inspection, I noted the tag # changed to (b) (6) [redacted]. Please confirm this tag number is correct.

I have no concerns with your Section T; your PI-managed housing SOP will be updated to reflect the new protocol number.

Thank you,
(b) (6) [redacted]

(b) (6) [redacted]
Office of Animal Welfare Assurance
Duke University

(b) (6) [redacted]
Durham, NC 27705
(b) (6) [redacted]
(b) (6) [redacted]
Web: https://dacup.duke.edu/

Duke University Institutional Animal Care and Use Committee (DUIACUC)

Occupational and Environmental Safety Office Hazardous Agent Review

PI: Heath G. Gasier | Dept/Div: Medicine / Pulmonary / Hyperbaric Center | Date Received: 11/09/2021

Project: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure

REG#: A224-21-11 | Housing Location: (b) (6) | OAWA Contact: (b) (6)

☒ **Biological Agents**

☒ Cleared For: BSL1 ☐ BSL2 ☐ BSL3 ☐ Transgenic/KO ☐ Micro-Organisms ☐ rDNA/IBC/IRE ☐

☐ Conditional Biological Toxins ☐ Human Material ☐

Comments:

NIH exempt breeding: mCherry-GFP-FIS1(101-152) in the mouse Rosa26 locus of C57BL/6 background.

Reviewed by: (b) (6) Date: 11/09/2021

☒ **Chemical Agents**

☒ Cleared Hazardous Chemicals ☒ Hazardous Drugs ☐

☐ Conditional Fixed Tissue ☐

Comments:

Isoflurane in a bell jar on 4x4 gauze below a raised platform to separate mice for contact with the isoflurane.
[CAS: 26675-46-7] GHS Lookup Tool. STOT-Repeated Exposure, Category 2. Narcotic Effects, Category 3. Hazardous. must be used with an effective mechanism of waste gas scavenging (see The Safe Use of Anesthetic Gases policy found on the Duke Animal Care and Use Program Policies webpage). https://dacup.duke.edu/safe-use-anesthetic-gases (login required). Follow safety guidelines for applicable hazards when using. If using a charcoal canister for scavenging, this must be submitted as chemical waste through OESO, not disposed in the trash. No special animal handling precautions.

Tiagabine (TGB, Gabitril)[145821-59-6]:
Not a hazardous substance or mixture (Sigma SML0035 SDS).

Reviewed by: (b) (6) Date: 11/09/2021

☐ **Radiation Sources**

☐ Cleared Isotopes ☐ X-Ray ☐

☐ Conditional Irradiator ☐ Laser ☐

Comments:

Reviewed by: Date:

☒ **Lab Audit**

☒ Cleared Next Audit Due: 12/17/2021

☐ Conditional

Comments:

Reviewed by: (b) (6) Date: 12/17/2020

OESO Departmental Approval

The referenced protocol has been reviewed and approved for the use of hazardous agents in animals as follows:

- [x] Cleared as written
- [ ] Conditional clearance pending implementation of all recommendations listed above.

Additional Comments

SC 81647: PI change from Piantadosi to Heath G. Gasier

OESO Representative: (b) (6) [redacted]

Date: 11/09/2021

(b) (6)

From: (b) (6)
Sent: Wednesday, November 10, 2021 2:05 PM
To: Dr Heath Gasier, Ph.D.
Subject: Review of Protocol A224-21-11 (Agenda 7N)
Attachments: Gasier Review A224-21-11 7N.docx

Dr. Gasier,

Please see the attached veterinary review of your recently submitted animal care and use protocol. You should reply **within the attached document**, **unless otherwise requested**, and send this back to me.
If you could have responses back by November 16, 2021, this will help facilitate review and approval of your protocol at the upcoming IACUC meeting.

Please let me know if there are any questions.

Thank you,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

To: Dr. Heath Gasier
Registry Number: A224-21-11
Agenda Sequence: 7N
Protocol Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure
Requested Action: Please consider and respond to me as soon as possible

The Duke Institutional Animal Care and Use Committee (IACUC) will review your animal use application shortly. In preparation for a smooth review process and with the hope for a more rapid approval of your proposal, please review and respond to the following veterinary review concerns. **All responses and documents related to this protocol should be sent via the principal investigator's "duke.edu" email account; this will serve as the electronic signature.**

CONCERN NUMBER 1. ROLE DELINEATION (A3)
Please clarify what procedures described in the protocol application that (b) (6) [redacted] will be responsible for.

CONCERN NUMBER 2. ANIMAL WELL-BEING (D6)
The narrative states that mice that show extended time of seizures will be euthanized, but it is unclear how long this is.
Please clarify what the maximum time of seizure activity that will be allowed prior to euthanasia.

CONCERN NUMBER 3. EUTHANASIA (E1)
Please confirm that all animals that undergo cervical dislocation under anesthesia will also have a visible confirmatory method performed (i.e. bilateral thoracotomy, decapitation).

CONCERN NUMBER 4. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the maximum volume administered for the IP Injections.

CONCERN NUMBER 5. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the method of restraint used for the tail vein blood collection procedures.

CONCERN NUMBER 6. MONITORING AND HUMANE ENDPOINTS (M12)
This section describes daily monitoring for 3 days post HBO2, but the protocol application describes studies where animals will be housed for more than 3 days post HBO2 exposure. Please clarify how animals are monitored for the entire duration that they are housed under the protocol, which is until euthanasia.
How often are animals that undergo multiple HBO2 exposures monitored between exposures?

CONCERN NUMBER 7. MONITORING AND HUMANE ENDPOINTS (M12)
Please confirm that animals are monitored continuously during the time that seizures are anticipated.

While these are the only clarifications requested from the veterinary review, the IACUC Primary Reviewer may contact you for additional clarifications. Please respond as quickly as possible within this document with your clarifications of the concerns identified in this document.

Please **DO NOT** amend your application for animal use, unless specifically requested to do so. Submission of an amended protocol at this stage of the process may impede the overall review, since it will require a complete re-review and may delay approval.

Thank you for your time and effort in this regard. We appreciate your partnering with us to secure a complete review package for the IACUC's consideration. Our goal is a more rapid approval process for your research laboratory, while assuring institutional regulatory compliance, as dictated by the Animal Welfare Act and the Public Health Service (NIH) policy.

Best Wishes,

(b) (6) [redacted]
Division of Laboratory Animal Resources
Duke University Medical Center

(b) (6)

From: (b) (6)
Sent: Tuesday, November 16, 2021 2:32 PM
To: Dr Heath Gasier, Ph.D.
Subject: RE: Review of Protocol A224-21-11 (Agenda 7N)
Attachments: Gasier Review A224-21-11 7N_Resolved.pdf

Hi Heath,

Thank you for the responses to the review.

The review concerns are resolved. I have attached the document to reflect this for your protocol records. This will also be placed in and become part of the medical record.

The protocol will be presented at the IACUC meeting this Thursday.

Thanks again,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

From: Dr Heath Gasier, Ph.D.
Sent: Thursday, November 11, 2021 9:15 AM
To: (b) (6)
Subject: RE: Review of Protocol A224-21-11 (Agenda 7N)

Good morning (b) (6)

Attached are the answers to your questions/concerns. Please let me know if you need more detail.

Thank you.

vr

Heath

From: (b) (6)
Sent: Wednesday, November 10, 2021 2:05 PM
To: Dr Heath Gasier, Ph.D. (b) (6)
Subject: Review of Protocol A224-21-11 (Agenda 7N)

Dr. Gasier,

Please see the attached veterinary review of your recently submitted animal care and use protocol.

You should reply **within the attached document**, **unless otherwise requested**, and send this back to me.
If you could have responses back by November 16, 2021, this will help facilitate review and approval of your protocol at the upcoming IACUC meeting.

Please let me know if there are any questions.

Thank you,
(b) (6)

(b) (6)
Division of Laboratory Animal Resources
Duke University Medical Center
Office: (b) (6)

To: Dr. Heath Gasier
Registry Number: A224-21-11
Agenda Sequence: 7N
Protocol Title: The Role of GABA in Susceptibility to Oxygen Toxicity with Repeated HBO2 Exposure
Requested Action: Please consider and respond to me as soon as possible

The Duke Institutional Animal Care and Use Committee (IACUC) will review your animal use application shortly. In preparation for a smooth review process and with the hope for a more rapid approval of your proposal, please review and respond to the following veterinary review concerns. **All responses and documents related to this protocol should be sent via the principal investigator's "duke.edu" email account; this will serve as the electronic signature.**

CONCERN NUMBER 1. ROLE DELINEATION (A3)
Please clarify what procedures described in the protocol application that (b) (6) will be responsible for.
Response: We initially were going to generate the (b) (4), (b) (6) [redacted] Because she will not be breeding them, here role will be minimal and consist of monitoring health and well-being, mice identifcation (ear tags), and assist with transportation to (b) (6)

CONCERN RESOLVED

CONCERN NUMBER 2. ANIMAL WELL-BEING (D6)
The narrative states that mice that show extended time of seizures will be euthanized, but it is unclear how long this is.
Please clarify what the maximum time of seizure activity that will be allowed prior to euthanasia.
Response: For 60 min exposures to HBO_2 at 5 ATA, mice are expected to have seizures that generally last several seconds. If a mouse has a tonic clonic seizure for >60 sec, the HBO_2 exposure will be terminated and mouse will be immediately euthanized using isoflurane.

CONCERN RESOLVED

CONCERN NUMBER 3. EUTHANASIA (E1)
Please confirm that all animals that undergo cervical dislocation under anesthesia will also have a visible confirmatory method performed (i.e. bilateral thoracotomy, decapitation).
Response: All mice are euthanized by isoflurane inhalation, followed by cervical dislocation which is a secondary method. This is always confirmed by bilateral thoracotomy since we need to obtain blood and lung tissue.

CONCERN RESOLVED

CONCERN NUMBER 4. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the maximum volume administered for the IP Injections.
Response: 5 µl per gram of body weight.

CONCERN RESOLVED

CONCERN NUMBER 5. NARRATIVE OF NON-SURGICAL PROCEDURES (M1)
Please provide the method of restraint used for the tail vein blood collection procedures.
Response: This will be a two person procedure. One person will restrain the mouse by placing their thumb and index finger on the dorsal neck and grasp the skin with one hand and extend the lower extremities with the other. The animal will be on a flat surface. The other person will collect the blood from the tail. This procedure generally takes 5-15 seconds.

CONCERN RESOLVED

CONCERN NUMBER 6. MONITORING AND HUMANE ENDPOINTS (M12)
This section describes daily monitoring for 3 days post HBO2, but the protocol application describes studies where animals will be housed for more than 3 days post HBO2 exposure. Please clarify how animals are monitored for the entire duration that they are housed under the protocol, which is until euthanasia.
How often are animals that undergo multiple HBO2 exposures monitored between exposures?
Response: Animals will be monitored twice daily (morning and late afternoon).

CONCERN RESOLVED

CONCERN NUMBER 7. MONITORING AND HUMANE ENDPOINTS (M12)
Please confirm that animals are monitored continuously during the time that seizures are anticipated.
Response: Mice are monitored from the time they enter the chamber until the time they are removed, encompassing the entire compression, HBO_2 exposure, and decompression periods.

CONCERN RESOLVED

While these are the only clarifications requested from the veterinary review, the IACUC Primary Reviewer may contact you for additional clarifications. Please respond as quickly as possible within this document with your clarifications of the concerns identified in this document.

Please **DO NOT** amend your application for animal use, unless specifically requested to do so. Submission of an amended protocol at this stage of the process may impede the overall review, since it will require a complete re-review and may delay approval.

Thank you for your time and effort in this regard. We appreciate your partnering with us to secure a complete review package for the IACUC's consideration. Our goal is a more rapid approval process for your research laboratory, while assuring institutional regulatory compliance, as dictated by the Animal Welfare Act and the Public Health Service (NIH) policy.

Best Wishes,

(b) (6) [redacted]
Division of Laboratory Animal Resources
Duke University Medical Center

Duke IACUC

From: Duke IACUC
Sent: Friday, November 19, 2021 9:30 AM
To: Dr Heath Gasier, Ph.D.
Cc: (b) (6) [redacted]
Subject: Approved - IACUC Meeting Results for Protocol A224-21-11
Attachments: 7N Gasier A224-21-11_Approved.pdf

Please see your attached approval letter for protocol A224-21-11.

For information on the responsibilities of a PI, please visit the program policy HERE

United States Department of Agriculture
Animal and Plant Health Inspection Service

MMCBRIDE
2016090000667812 Insp_id

Inspection Report

DUKE UNIVERSITY

Office Of Animal Welfare Assurance
2424 Erwin Rd Ste 606
DURHAM, NC 27705

Customer ID: **863**
Certificate: **55-R-0003**
Site: 001
DUKE UNIVERSITY

Type: ROUTINE INSPECTION
Date: 26-JUL-2021

No non-compliant items identified during this inspection.

This inspection and exit interview were conducted with facility representatives.

Additional Inspectors:

(b) (6) [redacted], VETERINARY MEDICAL OFFICER

Prepared By: (b) (6) [redacted]
USDA, APHIS, Animal Care

Date: 27-JUL-2021

Title: VETERINARY MEDICAL OFFICER

Received by Title: Registrant

Date: 27-JUL-2021

United States Department of Agriculture
Animal and Plant Health Inspection Service

Customer: 863
Inspection Date: 26-Jul-2021

Species Inspected

Cust No	Cert No	Site	Site Name	Inspection
863	55-R-0003	001	DUKE UNIVERSITY	26-JUL-2021

Count	Scientific Name	Common Name
000012	*Canis lupus familiaris*	DOG ADULT
000002	*Felis silvestris catus*	CAT ADULT
000062	*Macaca mulatta*	RHESUS MACAQUE
000026	*Sus scrofa domestica*	DOMESTIC PIG / POTBELLY PIG / MICRO PIG
000179	*Oryctolagus cuniculus*	DOMESTIC RABBIT / EUROPEAN RABBIT
000007	*Papio anubis*	OLIVE BABOON
000028	*Mustela putorius furo*	DOMESTIC FERRET
000014	*Macaca fascicularis*	CRAB-EATING MACAQUE / CYNOMOLGUS MONKEY
000040	*Mesocricetus auratus*	SYRIAN / GOLDEN HAMSTER
000370	**Total**	

SATELLITE ANIMAL FACILITY DISASTER RESPONSE PLAN

Research Integrity & Compliance
University of South Florida
email (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Completed forms should be saved as a PDF and uploaded into the ARC system viewable at:
https://ARC.research.usf.edu/prod/

Emergency/Disaster Response Plan for (b) (6)(b) (6)(b) (6) (Satellite Animal Facility Location e.g., Bldg., Room #)

1. Satellite animal facilities are locations where animals are maintained/housed >12 hours, which are not managed or attended to daily by Comparative Medicine staff, but instead are overseen by the Principal Investigator (PI) and research staff.

2. Any facility may be subject to unexpected conditions that results in the catastrophic failure of critical systems, significant personnel absenteeism, or other unexpected events that severely compromise ongoing animal care and well-being.

3. All animal facilities, including satellite animal facilities, must have a disaster plan.

4. This disaster plan has been established in conjunction with the PI directing animal activities within the satellite animal facility, **as an addendum to existing institutional plans and policies**, and takes into consideration personnel safety, the priorities for triaging animal populations, and institutional needs and resources.

5. This plan defines the actions necessary to ensure personnel safety and prevent animal pain, distress, and deaths, with consideration for potential **natural disasters** (e.g., flooding, tornado, hurricane, severe storms), **operational disasters** (e.g., loss of systems that control ventilation, cooling, heating, provision of potable water), and/or **security/technological disasters** (e.g., civil disturbance, biological or chemical hazards) and describes how, in the event of a disaster, the satellite facility will respond.

6. Veterinarians are provided access to animals housed in all satellite animal facility locations.

7. This plan will be reviewed by the IACUC and, upon its approval, will become part of the overall institutional disaster response plan coordinated by the IO.

8. **This addendum plan includes that**:

    a. Efforts will be taken to **ensure personnel safety** and security first during and immediately after a disaster, **followed by the safety and welfare of the animals** in our care.

    b. After ensuring personnel safety, **animals that are irreplaceable and necessary for research activities will be provided with daily care.** If campus lock-down is projected, consider whether offering supplemental feed and drinking water contributes to the long-term care of animals in the event access to the satellite facility may become limited.

    c. **Animals that cannot be cared for, or provided with supplemental feed and drinking water, or relocated, or protected from the consequences of the disaster will be euthanized**.

    d. Sufficient **preparation** will occur beforehand (e.g., staff understand the specific personnel and animal protective measures, the location of emergency response kits, and that the status of inventories of feed, water, sanitation, and protective supplies should be confirmed), as will **clear and immediate communication**, both of which are acknowledged as critical keys to successfully managing all disaster situations.

    e. Each individual listed below is **familiar with this plan**, evacuation routes, locations of telephones, fire alarms and extinguishers, first aid kits, emergency response kits, emergency shower and eyewash stations, and emergency supplies, as applicable.

f. In the event of an emergency, law enforcement and emergency personnel are able to reach the personnel responsible for animals, below, by **prominently posting in the satellite animal facility these emergency contact names and telephone numbers**, and will assist them with access.

SATELLITE ANIMAL FACILITY DISASTER RESPONSE PLAN

Research Integrity & Compliance
University of South Florida
email (b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
Completed forms should be saved as a PDF and uploaded into the ARC system viewable at:
https://ARC.research.usf.edu/prod/

Emergency/Disaster Response Plan for (b) (6)(b) (6)(b) (6) (Satellite Animal Facility Location e.g., Bldg., Room #)

g. This disaster plan identifies the following **essential personnel** who have been trained in advance in its implementation, and have completed **institutional requirements of essential personnel** to ensure access.

NAME (Please print)	**ROLE** (e.g., PI, research staff, bldg. mgr.)	**EMERGENCY PHONE #** (e.g., 24/7)
(b) (6)(b) (6)	PI	(b) (6)(b) (6)(b) (6)(b) (6)(b) (6)
(b) (6)(b) (6)	research staff, doctoral grad student	(b) (6)(b) (6)
Veterinarian on call	Veterinarian	(b) (6)

h. **Specific measures** that will contribute to personnel safety and the welfare of animals in our care in the event of a disaster are described below. In your response, **please indicate** that this emergency, disaster response plan (1) takes into consideration both personnel and animal safety during an emergency or disaster, (2) ensures satellite staff are institutionally enrolled as "essential personnel", (3) ensures that in the event of campus lock-down, the study will not be initiated, the satellite-housed animals will be preemptively provided with supplemental feed and drinking water, animals will be cared for daily, or the satellite-housed animals will be euthanized preemptively. (**Note: a statement is required**):

This emergency disaster response plan takes into consideration both personnel and animal safety during an emergency or disaster, and it ensures satellite staff are institutionally enrolled as "essential personnel". If an emergency occurred while mice are in the oxidative preconditioning chamber (Expt. 8) then the exposure will be terminated. If there is time to safely return the animal to the vivarium then that will be done. If, however, there is not sufficient time to return the animal then exposure will be terminated and the animal will be euthanized. In the event that the the event causes a campus lock-down, the study will not be initiated and all animals will remain in the COM vivarium. While in the P.I.'s lab, all animals are provided feed and drinking water.

RESEARCH PROPOSAL SUBMITTED TO THE OFFICE OF NAVAL RESEARCH

PROPOSAL TITLE: Assessment of Kv2.2 channels as therapeutic targets for oxygen toxicity in rats following HBO exposure

LABORATORY: Naval Medical Research Center
Undersea Medicine Department
503 Robert Grant Avenue
Silver Spring, MD 20910-7500

PRINCIPAL INVESTIGATOR: Aaron Hall, Ph.D.

CO-INVESTIGATOR(S):

USE OF HUMAN SUBJECTS (Y/N): N

USE OF ANIMALS (Y/N): Y rats

RECOMBINANT DNA RESEARCH (Y/N): N

PERSONNEL/ENVIRONMENTAL HAZARDS (Y/N): Y

PROPOSED START DATE: 1 JULY 2022

PROPOSED BUDGET ($):

	DIRECT	INDIRECT	TOTAL
FY22	(b) (4)	(b) (4)	(b) (4)
TOTAL	(b) (4)	(b) (4)	(b) (4)

I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports and agreed-upon products to the Office of Naval Research.

HALL.AARON.ANDREW.1392298068 Digitally signed by HALL.AARON.ANDREW.1392298068 Date: 2022.06.16 12:05:49 -04'00'

Principal Investigator Date
Phone: 301-319-7189
Fax: 301-319-7378
Email: Aaron.a.hall6.civ@mail.mil

(b) (6)

Approval Authority Date
Phone:
Fax:
Email:

(b) (6)

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C. Statement of Objectives:

Enriched oxygen breathing gases are used extensively in closed circuit under water breathing apparatus (CC-UBA) systems to increase stealth and reduce the amount of dive time dedicated to decompression. Prolonged exposure to hyperbaric oxygen (HBO) with a pO2 greater than 0.4ATA (140kPa) increases the prevalence of oxygen toxicity (1), which effects several tissue beds including: skeletal muscle, lung, and the CNS. These effects present as symptoms such as fatigue and tracheobronchitis (2). Manifestation of these symptoms can have a deleterious impact on diver safety, comfort, and mission effectiveness.

Voltage-gated potassium channels regulate cell function globally, with profound effects on cellular and system level homeostasis. The primary function of these channels is to both regulate cell membrane potential and to repolarize the cell membrane following an action potential (3). In a human study at NSMRL, the potassium chanel Kv2.2 was identified as functionally enriched in the blood after a 6.5hr, 2.0ATA HBO exposure. Kv2.2 has not been previously studied under hyperoxic conditions. Kv2.2 is an oxygen sensitive voltage gated potassium channel (4).

The **objective of this study** is to determine whether Kv2.2 function contributes to the manefestation of oxygen toxicity in rats and is thus a target for countermeasure development.

D. Statement of Hypotheses and Specific Research Aims:

We hypothesize that pharmacological/biological manipulation of Kv2.2 activity will modify oxygen toxicity symptoms associated with HBO exposure. Our specific research aims are:

1.) Determine effect of Kv2.2 antagonists on pulmonary oxygen toxicity in rats following a 6.5hr 2.0ATA HBO exposure.
2.) Determine the effect of Kv2.2 agonists on pulmonary oxygen toxicity in rats following a a 6.5hr 2.0ATA HBO exposure

E. Statement of the Approach:

(b) (6) has a well-established rodent HBO model which will be used for this study. Rats will have a baseline assessment of muscle and pulmonary function, be randomized to receive intervention, and then be compressed to 2.0 ATA on oxygen for 6.5 hours. Rats will then have functional assessment of muscle and pulmonary function and be euthanized for blood and tissue collection. This 2.0 ATA oxygen model was selected to mirror the conditions of the human study.

F. Statement of Significance:

One of the major limiting factors for Navy Divers utilizing rebreather diving rigs (MK 16 or MK 25) is limited bottom time due to the manifestation of oxygen toxicity symptoms. Increasing the capacity to utilize higher pO_2 during diving missions will reduce decompression obligations and improve mission capability by directly shifting a greater proportion of dive time away from decompression towards mission execution. Currently it is recognized that diver physiology is a limiting factor in diving operations. The current study seeks to identify new targets to enhance tolerance to HBO while simultaneously preserving diver safety and comfort.

G. Key Word/Phrase Listing:

Oxygen toxicity, hyperbaric oxygen, Voltage Gated Potassium Channels, Rodents.

H. Body of Proposal:

1. The Idea

Human studies conducted at NSMRL indicated that the Kv2.2 channel was upregulated in the serum of individuals exposed to a 2.0ATA hyperbaric oxygen exposure for 6.5 hours which induced symptoms of pulmonary oxygen toxicity. Kv2.2 has not been studied under conditions of hyperbaric hyperoxia. We propose to examine whether blockade or stimulation of the Kv2.2 changes the onset of pulmonary injury associated with HBO exposure.

2. Introduction and Background

Prolonged hyperbaric oxygen exposure produces symptoms in the lungs, central nervous system, and skeletal muscle. A study conducted at NSMRL exposed human subjects to a 2.0ATA hyperbaric oxygen exposure for 6.5hrs. Blood samples collected pre-dive and 1hr post-dive were analyzed for changes in protein content which demonstrated functional enrichment of 3 proteins, APOD, KCNB2 (Kv2.2), and VP13C (Figure 1). KV2.2 is an oxygen sensitive potassium channel subunit which assists with repolarization of membrane potential after action potentials in a variety of tissues including vascular endothelium, skeletal muscle, and brain. Due to this oxygen sensitivity, we propose to examine the role Kv2.2 channels play in the development of symptoms associated with HBO toxicity.

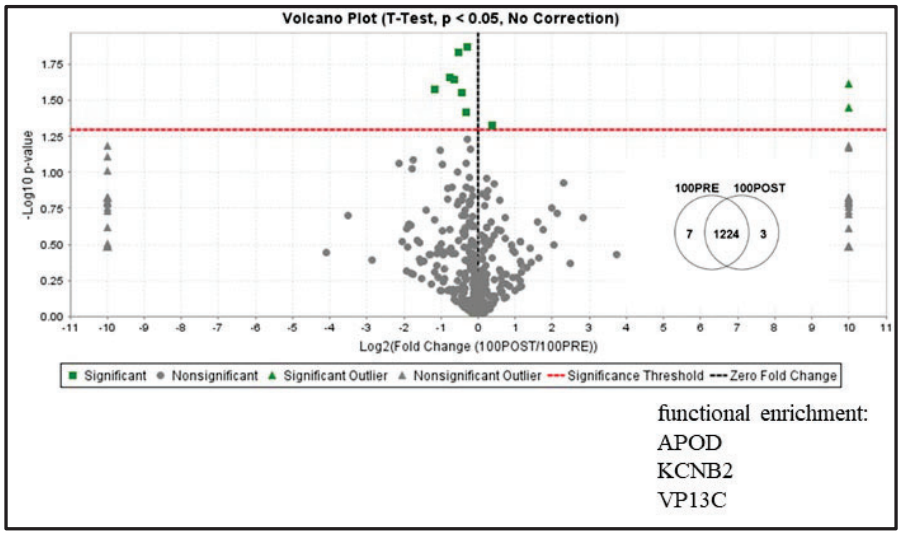

Figure 1: Volcano Plot showing functional changes in serum proteins following a 6.5hr 2.0ATA HBO exposure in human subjects

Kv2.2 channels belong to the Shab subfamily of potassium channel subunits and form both homodimers and heteromeric channels with potassium channel subunits from other subfamilies such as Kv8.2 (5). These channels regulate repolarization rates following action potentials and are involved in sleep/arousal (6), smooth muscle function (7), and pulmonary vascular function (8). In vivo, these channels are activated by nitric oxide (9) and have an oxygen sensitivity (4).

3. Technical Approach

To evaluate Kv2.2 channels as therapeutic targets for oxygen toxicity we will execute 2 aims of study. In aim 1, we will use the tarantula based toxin GxTx1E to antagonize the Kv2.2 channel *in vivo* prior to exposure to a 6.5hr 2.0ATA HBO exposure. In aim 2, we will indirectly activate the Kv2.2 channel using the guanylyl cyclase agonist Riociguat then antagonize Kv2.2 function using GxTx1E to determine the relative contribution of Kv2.2 to any effects on oxygen

toxicity observed. Due to gender differences observed with oxygen exposure and Kv2.2 expression/function both male and female groups will be studied.

Aim 1: Rats (n=48) will be randomized into 4 groups (n=12/group) consist of an equal number of male and female rats: Control Air, Control HBO, GxTx1E Air, GxTx1E HBO. Following standardized acclimatization, rats will have baseline functional assessments of peripheral muscle strength and pulmonary function. Rats will then receive toxin (or control) injection and be placed in a hyperbaric chamber. The rat will be pressurized to 2.0ATA, have their breathing gas switched to 100% oxygen, and be held for 6.5 hours. Following the 6.5hour exposure the rats will be returned to surface and have post-dive assessment of muscle and pulmonary function.

Aim 1: Rats (n=48) will be randomized into 4 groups (n=12/group) consist of an equal number of male and female rats: Control Air, Control HBO, Riociguat HBO, Riociguat+GxTx1E HBO. Following standardized acclimatization, rats will have baseline functional assessments of peripheral muscle strength and pulmonary function. Rats will then receive toxin (or control) injection and be placed in a hyperbaric chamber. The rats will be pressurized to 2.0ATA, have their breathing gas switched to 100% oxygen, then be held for 6.5 hours. Following the 6.5 hour exposure the rats will be returned to surface and have post-dive assessments of muscle and pulmonary function.

The following functional parameters will be measured in all rats over the course of the HBO exposure study:

1.) Peripheral muscular strength and fatigue resistance – This endpoint will determine how afferent signaling affects local muscular fatigue in the absence of central motor input by assessing time required to reach 50% of maximal tetanic force production in rodent hind limb muscle via electrical stimulation (120hz) of the motor nerve (central fatigue) or stimulation (120hz) of the motor end plate (peripheral fatigue). Somatosensory evoked potentials (SEP) will be recorded using a stimulator (Stimulator S 88, Grass, Quincy, MA) and EEG amplifier (Wide band A. C. pre-amplifier, 7P3c and Polygraph D.C. driver amplifier. Data will be sampled at a rate of 1 kHz on PC and exported for off-line analysis.

2.) Pulmonary function- This endpoint will determine how afferent signaling effects pulmonary function in un-anesthetized rats using whole body plethysmography. The specific endpoint used will be enhanced pause (Penh), which is a dimensionless unit that correlates with pulmonary resistance, in addition to minute ventilation, tidal volume, and respiratory rate. A hypercapnic challenge protocol will be utilized to evaluate ventilation responsiveness. This measurement will be measured using a whole-body plethysmograph (Buxco Electronics Ltd., NY) connected to a standalone PC running Fine-Point software and exported for off-line analysis.

Oxygen toxicity parameters 1 and 2 will be measured pre-dive and post-dive. After the functional assessment, the rats will be euthanized and histological examination of the gastrocnemius muscle and the lungs will be conducted.

4. Transition Plan

Once the described experiments are completed, the results will be submitted for publication in a peer reviewed journal. The findings from this research study will be shared with (b) (6) [redacted] at ONR in the form of regular research reports. Final results will also be shared with (b) (6) [redacted] to support the ongoing manned diving experiments utilizing the HBO model used in this study. Additionally, if one of the treatment groups demonstrates improved outcomes following HBO exposure, a follow on study will be submitted to ONR to transition these findings to a higher order animal model.

I. Facilities available:

The diving experiments outlined in this application will be performed at (b) (6) where all the necessary equipment is available and operational for use. These include:

A. Hyperbaric Labs Capability Summary

General: The hyperbaric laboratories (b) (6) [redacted] presently contain 7 hyperbaric chambers ranging in size from 0.5 to 30 cubic feet floodable volume. The chambers support Research Programs in Decompression Sickness and Oxygen Toxicity. Capabilities are available for single or multiple small animals as well as single or multiple large animals.

Depth Hull #	Floodable Volume	Pressure	Depth (fsw)	Depth (ATA)	Gas Capabilities
H-176-1 (H2)	5 CF	500 psig	1120	35	Air & Mixed Gas (HeO_2)
H-209	5 CF	500 psig	1120	35	Air & Mixed Gas (HeO_2)
H-192	20 CF	325 psig	730	23.0	Air Chamber , w/ O_2 or Air closed circuit overboard dump
H184 (H1)	5 CF	325 psig	730	23.0	Air, O_2, & N_2
H-140	5 CF	115 psig	258	8.8	O_2 & N_2
MLAC	450 CF	550 psig	1230	38	Air Chamber , w/ O_2, Air, or Mixed Gas closed circuit overboard dump
SSAC – (4 chambers)	.025 CF	35 psig	78	3.3	Air, O_2, N_2, Mixed Gas
SRC	.065 CF	35 psig	78	3.3	O_2 & N_2

Breathing Gas: Various configurations allow for many combinations of gas delivery. Some chambers require subjects to breathe directly from the chamber atmosphere, while others are designed with a closed circuit overboard dump breathing system. These systems allow switching of the breathing gas on the fly during diving operations independent of the chambers atmosphere.

Gas Analysis: Sample points throughout the systems monitor and control Oxygen and Carbon Dioxide in both the chamber atmosphere and the auxiliary systems.

Infusion: Venous and arterial infusion lines (for swine subjects) are available on surface or at depth.

Vital Signs / Life Support Parameters: Capabilities for swine include the ability to monitor pulse, respiration, blood pressure, EEG, EKG and rectal temp; monitoring O_2 and CO_2 of chamber atmosphere and exhaled breathing gases. Capabilities for rodents include EEG, EKG and O_2 and CO_2 of chamber atmosphere as well as exhaled breathing gases.

Environmental Control: Chamber temperature and humidity are monitored. Most chambers have provisions for limited control of these variables.

Exercise: Three of the 5 cu ft. chambers hold rotating exercise cages for multi-rat studies. Additional rotating cages are available on the bench (1 ATA). A treadmill is available for large animals.

B. General Laboratory Space:

In addition to the hyperbaric chamber facility, there is a fully equipped laboratory which contains a water bath, luminometer, low- and high- speed centrifuges; FACS-STAR 5 flow cytometer, phosphoimager and densitometer, ELISA washer/plate reader, gas chromatograph/mass spectrometer. Animals will be housed at the AAALAC certified facilities

described above under the care and supervision of the Institution's Laboratory of Animal Medicine and Science Division.

C. Animals:

Animals will be housed at the above facilities under the care and supervision of the Institution's Laboratory of Animal Medicine and Science Division.

J. Annual Budget ($):

SUMMARY

Item / Category	FY 2022
Personnel	(b) (4)
Equipment and Maintenance	
Expendable Supplies	
Animal Purchase and Per Diem	
Travel	
Contracts/Consultants	
Other Direct Costs	
Total Direct	
Indirect	
TOTAL BUDGET	
Budget Estimate:	

DETAILS FOR FY22

Personnel	Title	% Effort	Cost
(b) (6)	Research Physiologist	10	(b) (4)
	Senior Scientist	5	
	Post-Doctoral Fellow	10	
	Chamber Technician	5	
	Chamber Technician	10	
	Animal Technician	10	

(b) (6)	Animal Technician	10	(b) (4)
	Statistician	5	
Total personnel cost			

Equipment and Maintenance	**Cost**
Chamber maintenance	(b) (4)
Chamber Specific Equipment/Fabrication	
Large Equipment Purchase (>3K)	
Total Equipment and Maintenance Cost	

Expendable Supplies	**Cost**
Consumable lab supplies	(b) (4)
Oxygen	
Total Expendable Supplies Cost	

Animal Purchase and Per Diem	**Cost**
96 rats	(b) (4)
Animal per diem	
Total Animal Purchase and Per Diem Cost	
	(b) (4)

Travel	**Cost**
Program Review	(b) (4)(b) (4)
Total Travel Cost	(b) (4)(b) (4)

Contracts/Consultants	**Cost**
Publication Costs	(b) (4)(b) (4)
Fees (Miscellaneous)	
Total Contracts/Consultants Cost	(b) (4)(b) (4)

Total Direct Cost	(b) (4)
Indirect Cost	

TOTAL FY22 COST	(b) (4)(b) (4)

K. Full Curricula Vitae

Please see attached.

L. Current and pending support of each investigator:

Aaron Hall:

1.) The impact of carbon dioxide on simulated DISSUB survival and decompression sickness at 5ATA air in a 70kg swine model. NAVSEA FY21-23 (delayed). Total Funding: (b) (4) , (b)(3)|Title 1 (b)(3)|Title 1 0.2 FTE
2.) T&E of Submarine Rescue Systems Decompression Plan. DHA 6.7, FY22-26. Total Funding (b) (4) , (b)(3)|Title 1 (b)(3)|Title 1 (b)(3)|Title 1 (b)(3)|Title 1 0.25 FTE
3.) Breath Test for Pulmonary Oxygen Toxicity, USAMMDA, FY22-23. Total Funding (b) (4) , (b)(3)|Title 10 (b)(3)|Title 10 (b)(3)|Title 10 (b)(3)|Title 10 0.5 FTE

M. Other agencies to which this proposal has been or will be submitted: none

N. Special information regarding certain types of experiments:

The present proposal involves the use of experimental animals. All research involving animals will be performed at the (b) (6) facility that has AAALAC accreditation and all regulations and laws will be fulfilled. Documentation of Institutional Animal Care and Use Committee (IACUC) approval for the animal use protocol will be forwarded under separate cover.

O. Appendices

	POSITION TITLE
(b)(3)[Title 10 USC 130B], (b) (6) (b)(3)[Title 10 USC 130B], (b) (6)	(b) (6), (b)(3)[Title 10 USC 130B] (b) (6), (b)(3)[Title 10 USC 130B] (b) (6), (b)(3)[Title 10 USC 130B] (b) (6), (b)(3)[Title 10 USC 130B]

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
(b) (6)	BS	2002	Microbiology/cell science
	MS	2005	Medical Sciences
	PhD	2009	Medical Sciences

Please refer to the application instructions in order to complete sections A, B, and C of the Biographical Sketch.

PUBLICATIONS

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ABSTRACTS

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EXPERIENCE/EMPLOYMENT

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References:

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UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

Date: Wednesday, February 12, 2020 2:43:08 PM

Print Close

Study Identification

1.1

The title and narrative of this IACUC application must match the title and narrative of the grant that supports this research.

Submit a separate IACUC application for each federal or major agency grant.

1.1.1 * **Main Title** >>Get Help

Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels

1.1.2 **Additional Titles** >>Get Help

1.1.3 * **Principal Investigator:**

The applicant Principal or Secondary Investigator must have a faculty appointment with the University or an appropriate appointment with the James A. Haley Veteran's Administration Medical Center, H. Lee Moffitt Cancer Center and Research Institute, or New College of Florida.

(b) (6)

1.1.4 **Secondary Investigator(s):**

First Name	Last Name	Organization	Profile
There are no items to display			

1.1.5 **Research Staff:** >>Get Help

List all IACUC-certified personnel that will contribute to the study.

Last Name First Name	Organization	Profile
(b) (6)	Morsani College of Medicine	(b) (6)
	Molecular Pharmacology and Physiology	(b) (6)
	Integrative Biology	

1.1.6 * **List Secondary Study Contact(s):**

Secondary study contacts can manage the correspondence of a protocol in the absence of the PI. >>Get Help

Person	Organization
(b) (6)	Molecular Pharmacology and Physiology

1.1.7 * **Please Select:**

● **New Project**

○ 3rd year renewal replacing a previously approved protocol

Study Personnel Certification Information

The following individuals' certification information must be current before IACUC approval can occur.
Please Note: The information on this form is read-only. An individual's certification information can be updated on his or her Researcher Profile. Verification dates are granted by the IACUC following review of submitted information

Principal Investigator:

Name	CV	Animal Experience		Documentation of Online Training Completion	Date Verified	Link to Profile
(b) (6) (b) (6)	CV August 2018_short form_(b) (6) (b) (6) USF Tampa.docx(0.01)	Rat	37	(b) (6) USF ARO 2012 05-24.pdf(0.03)	8/29/2015	(b) (6)
		Snake	2			
		Cat	5			
		Mouse	0			

Secondary Investigators:

Name	CV	Animal Experience	Documentation of Online Training Completion	Date Verified	Link to Profile

There are no items to display

Research Staff:

Name	CV	Animal Experience		Documentation of Online Training Completion	Date Verified	Link to Profile
(b) (6) (b) (6)	Resume(0.01)	Mouse	1	Animal Surgery Training.pdf(0.02)	8/26/2015	(b) (6)
		Rat	3			
(b) (6) (b) (6)	(b) (6) Biosketch(0.01)	Rat	29	AALAS Training 2012(0.02)	9/14/2015	(b) (6)
		Rabbit	4			
		Mouse	6			
		Chinchilla	3			
		Dog	5			

Name	CV	Animal Experience	Documentation of Online Training Completion	Date Verified	Link to Profile
(b) (6) (b) (6)	(b) (6) (b) (6) Resume 2-10-15 (1).pdf(0.01)	Rat 5	AALAS Certificate.jpg(0.04)	9/11/2015	(b) (6)

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Funding Information

2.1

2.1.1 * **Select the funding source which best describes the sponsor of this research (check all that apply):** >>Get Help

Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.)

2.1.2 * **Does the funding source/sponsor of this study require compliance with 21 CFR 58 Good Laboratory Practices (GLP) Standards?** >>Get Help

○ Yes ● No

2.1.3 * **Will this protocol use any VA Resources?**

○ Yes ● No

2.1.4 * **Will this protocol be supported by funds administered by Moffitt Cancer Center?**

○ Yes ● No

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Funding - Federal/Major Agency

2.2

2.2.1 **Indicate the federal agencies that award grants based on peer-reviewed proposals:** >>Get Help

Office of Naval Research

2.2.1a **Other federal or major agency:**

2.2.2 * **Please enter the grant or contract number:**

proposal requested by ONR Program Officer, in preparation, to be submitted Nov 2019 review

2.2.3 * **Upload the federal or major agency grant narrative, or portions that include the face page and research plan which describes the specific**

aims and methods involving vertebrate animal use. >>Get Help

Name	Modified	Version
(b) (6) _USF_Research Plan_ONR cover page and ojectives FY2020.pdf	11/8/2019 8:17 PM	0.01

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Protocol Type

3.1

3.1.1 * Select the option that best describes your protocol type: >>Get Help

Type
● Research or Teaching
○ Wildlife Research
○ Murine Colony Only
○ Antisera Production (Rabbit Only)
○ Tissue Use Only

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Alternatives and Literature Search

4.1

4.1.1 * On this page indicate that a search for alternatives and alternative methods has been conducted, including a search of at least two relevant databases (e.g., PubMed), covering the dates searched (i.e. at least the last ten years), using the indicated search term(s), and that the search was conducted on the indicated date. Two entries must be made to section 4.1.1, then respond to the following three sections. >>Get Help

Name of Database	Dates Covered	Key Words and Search Strategy	Date Search Conducted

	Name of Database	Dates Covered	Key Words and Search Strategy	Date Search Conducted
View	PubMed	1980-2019	(Objectives 1-3) mitochondria-targeted antioxidant therapy, MitoQ or Mitoquinone, brain slice, hyperbaric oxygen or HBO or HBO2, seizure, oxygen toxicity, rat; (Objective 4) aquaporin-4, gas channel, oxygen toxicity, CO2 retention, hyperbaric oxygen or HBO or HBO2, seizure, AQP4 knockout mouse; (Objectives 1-4) alternative, anesthesia, pain, radio-telemetry, brain slice	11/7/2019
View	Google Scholar	1980-2019	(Objectives 1-3) mitochondria-targeted antioxidant therapy, MitoQ or Mitoquinone, brain slice, hyperbaric oxygen or HBO or HBO2, seizure, oxygen toxicity, rat; (Objective 4) aquaporin-4, gas channel, oxygen toxicity, CO2 retention, hyperbaric oxygen or HBO or HBO2, seizure, AQP4 knockout mouse; (Objectives 1-4) alternative, anesthesia, pain, radio-telemetry, brain slice	11/7/2019

4.1.2 * **Indicate in a narrative below that in the search that non-animal alternatives were considered, that less sentient species were considered, and that none were found suitable. (The narrative must demonstrate that alternatives to the use of animals as described in this application are not available.)** >>Get Help

A search for non-animal alternatives and a less sentient species was conducted. However, this is the first study to ever study the effects of MitoQ on real time production of reactive oxygen and nitrogen species (RONS) and electrophysiology in the context of hyperbaric oxygen therapy, diving and submarine medicine. Likewise, to the best of our knowledge, we are the only laboratory currently studying the cellular mechanism of oxygen toxicity and the interactions between O2 and mitochondria-targeted antioxidant therapy using fluorescence imaging, electrophysiology, and radio-telemetry under hyperbaric conditions. It would be premature to employ alternative non-animal models. Computer simulation would be inappropriate until the fundamental properties of these neural networks under hyperoxic conditions are identified using in vitro (brain tissue slices) electrophysiological and imaging methods and experimentation such as we have proposed in this petition. Likewise, immortal cell lines would also be inappropriate until the mammalian CNS has received sufficient study using tissue slice techniques. While tissue cultures of cells could also be used, it would not be wise to use them exclusively since previous work in my lab, and by others, have shown that cellular properties can vary widely between the acute tissue slice and chronically cultured cells.

4.1.3 * **Indicate in a narrative below that alternatives to painful procedures were considered, none were found suitable, and that methods described will be continuously refined so as to reduce animal discomfort. (The narrative must demonstrate that alternatives to these procedures as described in this application which may cause pain or discomfort to animals are not available or suitable.)** >>Get Help

For some experiments, we will euthanatize older rat pups and recently weaned rats using rapid decapitation without CO2 anesthesia. The brain is dissected free and prepared for harvesting living brain tissue slices for electrophysiology and microscopy studies. PLEASE NOTE: we previously studied the effects of CO2 narcosis/anesthesia on the data collected in rat brain slices. Our findings (published) show that using CO2 narcosis prior to rapid decapitation affects certain neurological phenomena in the caudal solitary complex neurons that are the subject of our research. Specifically, we have found that it alters the interaction between CO2 and hyperoxia and the specific redox reactions that occur. For this reason, we have elected NOT TO USE CO2 anesthesia/narcosis

in this new animal use protocol. In other experiments, animals will be anesthetized for sterile surgery and treated with analgesics pre- and post-surgically to minimize pain from telemetry module and recording probe implants.

4.1.4 * **Indicate in a narrative below that this animal use does not unnecessarily duplicate previous research or existing studies:** >>Get Help

Our search revealed that this research does not duplicate prior research or ongoing studies. Only 3 hits occurred when combining "mitochondria-targeted antioxidant therapy" and "hyperbaric oxygen" and these dealt with Parkinson's Disease, high altitude exposure, and a review article on the role of oxidative species in cell signaling; that is, there was no scientific overlap. Likewise, combining "aquaporin 4" and "hyperbaric oxygen" resulted in 4 hits and none of them overlapped scientifically with the proposed study. All other combinations of searches did not provide evidence of overlapping research.

Characteristics of Animals and Pain Category of Research

List and describe the animals to be used. Indicate the anticipated number of animals to be used in each Pain Category of Research and the total number of animals involved during the 3-year approval period of this protocol. Indicate strain or line designation if rodents are requested. Indicate the number of each species/strains that will be involved in planned procedures that are anticipated to produce momentary, slight, or no pain, discomfort or distress (Pain Category A); more than momentary or slight pain, discomfort or distress which is alleviated by the use of appropriate anesthetics and/or analgesics (Pain Category B); or pain, discomfort, or distress, which cannot, or is not alleviated by the administration of appropriate anesthetics and/or analgesics (Pain Category C). (Note: Refer to IACUC Principles & Procedures XII.)

5.1.1 * **For each animal group, click Add and complete the form.**

	Species / Stock/Strain / Characteristics	# Pain Category A	# Pain Category B	# Pain Category C	Total Number
View	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male)	0	0	50	50
View	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)	0	0	305	305
View	Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)	0	0	20	20
View	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)	122	0	0	122
View	Rat: Sprague-Dawley (neonates & weaned rats born but not used in this research (too old for certain experiments) but made available to other researchers on different protocols or euthanatized if unwanted)	13	0	0	13

	Species / Stock/Strain / Characteristics	# Pain Category A	# Pain Category B	# Pain Category C	Total Number
View	Rat: Sprague-Dawley (timed-pregnant or nursing dams)	12	0	0	12

If any additional strains have been added at Amendment, they will be shown below:

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Housing and Use Sites

6.1

6.1.1 * **Will animals be housed in a University-managed animal facility?**

● **Yes** ○ No

6.1.2 * **Will animals be transferred from the housing room to any animal research/procedural/testing areas outside of the University-managed animal facility?**

● **Yes** ○ No

6.1.3 * **Will any animals be housed at another institution?**

○ Yes ● **No**

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Housing Facilities

6.2

6.2.1 * **For each HOUSING FACILITY where animals will be housed, click Add and complete the form.** >>Get Help

Location Species

View	(b) (6) (b) (6) (b) (6)	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male) Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)

Location	Species
View (b) (6) (b) (6) (b) (6)	Rat: Sprague-Dawley (timed-pregnant or nursing dams) Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male) Rat: Sprague-Dawley (neonates & weaned rats born but not used in this research (too old for certain experiments) but made available to other researchers on different protocols or euthanatized if unwanted) Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)

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Outside Housing and Use Sites

6.3

6.3.1 * Enter the animal research/procedural/testing location where animals will be taken during the course of study, whether survival surgery will be performed, whether animals will be maintained/housed for >12 hours at this site, whether animals will need to be returned to a Comparative Medicine-managed facility after the procedure or that the procedure will be terminal, and provide a scientific or logistical justification for why animals must be removed from the facility: >>Get Help

Building	Room	Species	Survival Surgery	Housed >12 Hours	Return to Housing	Justification	Transport Method

	Building	Room	Species	Survival Surgery	Housed >12 Hours	Return to Housing	Justification	Transport Method
View	(b) (6)	(b) (6)	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male) Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)	no	no	yes	AQP4-KO & WT mice are transferred from the vivarium to (b) (6) so that they can be dived in while breathing hyperbaric oxygen to determine their relative risk for developing oxygen toxicity seizures. The hyperbaric chamber that is used to administer hyperbaric oxygen is installed in the P.I.'s lab and cannot be moved. Mice are returned to the vivarium after recovery from seizures and dived a maximum of 4 times with at least 1 wk between dives the result in seizures.	Mice are transported from (b) (6) (b) (6) across the courtyard (b) (6) (b) (6) in a vivarium-supplied cage atop a smoothly rolling cart. The animal cage is covered by 1-2 lab coats. The trip takes ~1.5 minutes.

	Building	Room	Species	Survival Surgery	Housed >12 Hours	Return to Housing	Justification	Transport Method
View	(b) (6)	(b) (6)	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male)	no	yes	yes	In Experiment 8, WT mice undergo 24h of oxidative preconditioning to elevated AQP4 expression in the brain prior to diving the animal in HBO to the endpoint of 1st and 2nd bouts of seizures. 24h oxidative preconditioning consists of four 90-min exposures to 2.5 ATA HBO interspersed by 4.5 h of exposure to 1 ATA air. Two mice are preconditioned at a time and remain in the sealed pressure chamber for 24h. Four and 1/2 h after the 4th preconditioning HBO exposure the mouse is transferred to the dive chamber and dived in 5 ATA HBO until onset of seizures. After that experiment, the 2nd mouse is dived also to seizure.	Mice are transported from (b) (6) (b) (6) across the courtyard to (b) (6) (b) (6) in a vivarium-supplied cage atop a smoothly rolling cart. The animal cage is covered by 1-2 lab coats. The trip takes ~1.5 minutes.

	Building	Room	Species	Survival Surgery	Housed >12 Hours	Return to Housing	Justification	Transport Method
View	(b) (6)	(b) (6) (b) (6) (b) (6)	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)	yes	no	yes	Animals are transported to PI's lab for one of three reasons. 1) To be used to acutely harvest brain tissue slices for in vitro experiments. Special equipment in the lab is used to keep the slices alive immediately following euthanasia, dissection, tissue slicing and incubation. 2) Rats are also brought to the PI's lab for recovery surgery; that is, to implant deep intracerebral recording electrodes & a radio-telemetry module that is connected to the deep intracerebral electrodes. Equipment in the lab is used to establish the electrodes are working while anesthetized before the animal recovers and is returned to the vivarium for later use in an experiment (at least 1 wk later). 3) Rats are also brought to the PI's lab to be dived in the hyperbaric chamber to assess their sensitivity to HBO and CNS-OT.	Rats are transported from (b) (6) (b) (6) across the courtyard to (b) (6) (b) (6) in a vivarium-supplied cage atop a smoothly rolling cart. The animal cage is covered by 1-2 lab coats. The trip takes ~1.5 minutes.

Study Summary and Animal Use Rationale

7.1

7.1.1 * **Very briefly summarize (less than 150 words) in lay terms that are intelligible to a nonscientist (1) the purpose of the study, (2) the sequential procedures involved (i.e., in general terms), and (3) why the study is important to human or animal health, the advancement of knowledge, or the good of society:** >>Get Help

The first purpose of this study is to determine if mitochondria-targeted antioxidant therapy decreases risk for central nervous system oxygen toxicity (CNS-OT; i.e., seizures) during exposure to hyperbaric oxygen (HBO). Mitoquinone (MitoQ) is the antioxidant to be tested. The second purpose is to determine if Aquaporin-4 (AQP4) gas channels are a contributing factor to O2 and CO2 uptake during exposure to HBO and CO2. Whole animal (rat & mice) and brain slice experiments (rat) will be done. Methods used include survival surgery (rat) to implant telemetric deep intracerebral electrodes; simulated “dives” in HBO using a pressure chamber (rat & mouse); tissue harvesting (rat) to measure levels of MitoQ, redox status, reactive species production, and brain cell electrical activity; tissue harvesting (mouse) to measure AQP4 levels. The study is important to determine if MitoQ mitigates risk of CNS-OT and to determine if AQP4 is a target for neuroprotection during exposure to HBO.

7.1.2 * **Provide a narrative that indicates the design of this study considered the impact of the proposed procedures on the animals' well-being and balance that with the specific aims of research. (Explain how the scientific benefits of the intended research outweighs harm to the animals):**

The design of this study considered the impact of the proposed procedures on the animals' well being relative to the scientific benefits. Two procedures are proposed in this animal use protocol: 1) Euthanasia by rapid decapitation, without anesthesia, to harvest living brain tissue slices for electrophysiological & fluorescence imaging experiments. CO2 narcosis/anesthesia is not used because we've reported (published) that CO2 alters the cellular responses to O2 and CO2 stimuli. 2) Stereotaxic neurosurgery &/or body cavity surgery in anesthetized animals (sterile, w/recovery) to implant radio-telemetry modules that are connected to deep intracerebral recording electrodes to measure brain activity in the brainstem and forebrain in awake animals. These types of procedures and experiments are necessary at this time because very little is known about the the sites for seizure genesis in the brain and the cellular mechanisms by which HBO induces seizures. Moreover, it is unknown if mitochondria-targeted antioxidant therapy provides neuroprotection against CNS-OT seizures. Thus, these experiments provide important, new information as the U.S. Navy works to develop mitigation strategies for using HBO in the undersea soldier for longer and safer diving operations. Research done in small animal studies at USF has been/will be used to provide guidance to U.S. Navy researchers using more sentient species (20 kg swine) and ultimately, U.S. Navy SEALs.

7.1.3 * **Describe the characteristics of each species/strain requested that justify its use in this protocol. If a specific sex is required, explain why:**

We will use the Sprague Dawley rat, both sexes, during the preweanling period (neonates) and shortly after weaning, which occurs around postnatal (P) day, P21. All of our prior research on O2, CO2 sensitivity and mechanisms of CNS-

OT has been done in this animal model. The young animals are used because their brain tissue is less optically dense and optimal for our proposed imaging techniques. Because the young animals exhibit similar physiological responses to hyperoxia and hypercapnia as adults they are an appropriate model for our experimental questions. Older animals are used in radio telemetry experiments because they are better suited for implanting the large 4ET telemetry modules. Aquaporin-4 knockout mice are used to test if AQP4 is important in oxygen and carbon dioxide uptake at the blood-brain barrier during exposure to hypercapnic HBO. Wild Type C57/BL6 mice express AQP4 and are the controls for the AQP4-KO mice. Males are used for all HBO diving experiments because males are more resistant to HBO induced seizures compared to females.

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Experimental Design

8.1

8.1.1 * **Provide a clear and concise description of the specific experimental manipulations and treatments of animals in chronological order so that IACUC reviewers understand exactly what will be done to all animals from entry into the study to its endpoints:** >>Get Help

BEGIN WHOLE ANIMAL RAT EXPERIMENTS EXPERIMENT SET 1) Determine the appropriate route and dose of MitoQ to administer to rats. OVERVIEW: This experiment has two parts. FIRST, acute toxicity testing is done to establish the safe dose of MitoQ as given by three different parenteral delivery routes: intravenous (i.v.), intranasal (nas.), and intramuscular (i.m.). SECOND, once the safe dose of MitoQ for each route of delivery (i.v., nas., i.m.) is determined, then the levels of MitoQ accumulation in the brain, blood and lungs will be determined as a function of time after dosing: times 0, 0.5, 1, 2 & 4 hr post-injection. Tissues harvested postmortem will be analyzed using liquid chromatography-dual mass spectroscopy. OUR GOAL is to identify the best route and dose that elevates brain levels of MitoQ within <1 to 2 hr. EXPERIMENT SET 1 IS NECESSARY because to date, MitoQ has been delivered to rats by adding it to their drinking water for 2-8 weeks. When MitoQ was delivered in this manner, it reached steady state levels in the brain and other tissues after 7-10 days. Once stopping treatment, MitoQ was quickly washed out (half-life = 1.5 days). In mice, however, a single i.v. injection (tail vein) of MitoQ in Swiss-Webster mice elevated levels in brain tissue in <1 hr with >90% clearance after 24 hr. Extrapolation of dosages from mice to rats is challenging since mice have a higher metabolism and thus often require a higher dose of test substance relative to that used in rats. This issue, however, will be quickly resolved using the up-and-down acute toxicity method in which the safe dose of substance can usually be achieved testing 6-10 animals (Lipnik R.L. et al. (1995) Comparison of the Up-and-Down, Conventional LD50, and Fixed Dose Acute Toxicity. Procedures. Fd. Chem. Toxic. 33(3): 223-231; and Dixon (1991) Staircase Bioassay: The Up-and-Down Method. Neuroscience & Behavioral Reviews. 15: 47-50). Up-and-Down Acute Toxicity Testing: based on the literature, 6-10 rats/delivery route will be needed to determine the safe vs. toxic dose of MitoQ. Using the highest number, we estimate that 30 rats will be needed to test 3 routes of delivery: [(3 routes: i.v., nas., i.m.) x (1 MitoQ) x (10 rats max. /route)] = 30 rats Liquid Chromatography Analyses: Once the upper limit for safe dosing with MitoQ is determined, we will then quantify its levels in brain tissue for each delivery route (i.v., nas. & i.m.) using liquid chromatography-dual mass spectroscopy. Animals will be dosed with MitoQ and then blood samples, lung samples, and brain regions (brain stem, hippocampi & cerebral motor cortices) harvested at 0.5, 1, 2 & 4 hr post-injection of MitoQ to measure levels of MitoQ. A fifth group of control animals without MitoQ treatment will be euthanized to collect baseline levels of MitoQ (i.e., time 0 and presumably zero levels of MitoQ) and to assess blood redox status. Animals are anesthetized using 3-5% isoflurane and an arterial blood sample taken (cardiac

puncture under isoflurane anesthesia) to measure the rat's redox status using the redoxSYS analysis meter (40uL blood required per sample for analysis). The animal is then euthanized and brain tissues are dissected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Based on a previous study, we plan to use six animals per group: [(6 rats/group) x (3 groups: i.v., nas., i.m.) x (1 substance: MitoQ) x (4 time points: 0.5, 1, 2 & 4 hr post-MitoQ injection)] + (6 rats: baseline group, time 0 (no MitoQ injection)) = 78 SD rats EXPERIMENT 2) Expose freely behaving rats to HBO2 -/+ MitoQ. OUR GOAL is to determine the effect of MitoQ on the latency time to onset of visible seizures. Unoperated rats (without radio-telemetry modules implanted) will be dosed with MitoQ, TPMP (triphenylphosphonium cation--the molecule that is bonded to coenzyme Q to render MitoQ highly permeable to the cell membrane and mitochondrial membrane) or vehicle (soybean oil) using the protocol that provides the highest level of MitoQ in brain tissues without toxicity (determined in Experiment 1). In parallel with MitoQ/vehicle dosing, each rat is adapted daily to the hyperbaric chamber, 1 hr/day x 7 days, before being dived on HBO2. After treatment with MitoQ, a single rat is placed in the animal chamber that is housed inside the hyperbaric chamber. The animal chamber is ventilated with 1 atmosphere absolute (ATA) air for at least 30 min before switching to 1 ATA O2 for another 15 min (note: 1ATA pressure = sea level = room pressure). The animal is then dived (~1 atmosphere gauge pressure, ATG/min) to 5 ATA O2. The animal is held at maximum depth until onset of first and second bouts of visible tonic-clonic seizures, which are separated by an interictal period that can range for tens of seconds to tens of minutes. The maximum time exposed to HBO is 2 hours; if seizures have not occurred then the HBO exposure is terminated. Thus, the maximum total time inside the chamber is 2h, 45min. Each animal has access to 5-6 pieces of food kibble lying on the floor of the animal chamber and a Hydrogel packet for drinking. The intensity of each bout of visible seizures is scored using our six-stage scale for CNS-OT seizures. Following the second bout of seizures, the animal is changed over to air and decompressed back to sea level (~1 ATG/min). Blood samples will be taken immediately following the dive (cardiac puncture under anesthesia) to measure the animal's redox status using the redoxSYS analysis meter (40uL/sample). Based on our past studies, we plan to use 10 rats/group: [(10 rats/group) x (3 treatments: MitoQ, TPMP & vehicle) = 30 SD rats. EXPERIMENT 3) OUR GOAL is to determine so called "ox tox trigger zones" where seizures originate during exposure to HBO and then determine the effects of mitochondria-targeted antioxidant therapy on this neural activity using MitoQ. Two to 4 intracerebral recording microelectrodes (tungsten; FHC, Inc.) are implanted using the stereotaxic frame/manipulator into motor cortex and deep subcortical nuclei that are implicated in seizure genesis (e.g., brain stem cardiorespiratory nuclei, cranial nerve nuclei, or medullary reticular formation) and seizure amplification/propagation nuclei (e.g., CA1 hippocampus, thalamus, or hypothalamus). Up to four recording electrodes can be implanted in the brain in the rat during sterile recovery surgery. Recording and reference electrodes are secured to the skull using dental adhesive. The telemetry module (4ET, Data Sciences International, DSI Inc.) is implanted in the abdomen and the four pairs of biopotential leads are tunneled under the skin, exposed at skull, and attached to the recording microelectrodes and epidural reference electrodes (Weiergräber et al. 2005 Electrocorticographic and deep intracerebral EEG recording in mice using a telemetry system. Brain Res 14(3):154-64; and Papazoglou et al. 2016 Non-restraining EEG Radiotelemetry: Epidural and Deep Intracerebral Stereotaxic EEG Electrode Placement. J Vis Exp. (112): 54216, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4993274/bin/jove-112-54216.mp4). After recovery from surgery (at least 1 wk), each animal is dosed with either MitoQ, TPMP, or vehicle and dived to 5 ATA O2 (HBO) until onset of 1st and 2nd bouts of seizures as in EXPERIMENT 2. After the first dive and bout of seizures, at least one week later, the animal is treated with one of the other two substances and dived again until onset of 1st & 2nd bouts of seizures. The order of treatments is randomized across two dives in animals; however, each rat is treated with only two substances and only dived twice, thus there are three test groups: MitoQ & TPMP, MitoQ & Vehicle, and TPMP & Vehicle. Each electrode records field potentials; i.e., neural activity from many neurons and synapses concurrently as their summed electrical activities. Recordings made in the brain stem (medulla oblongata, pons or midbrain) will be called an "electrobulbogram (EBG)"; in the thalamus or basal ganglia (caudate, putamen, globus pallidus & substantia nigra) will be called a deep "electroencephalogram (dEEG)"; and in the motor cortices it will be called an "electrocorticogram (ECoG)". All experiments are done in unrestrained, freely behaving animals.

The length of time spent in the animal chamber inside the hyperbaric chamber is no longer than 2h, 45min. Each animal has access to 5-6 pieces of food kibble lying on the bottom of the chamber and hydrogel. A PILOT STUDY will be done initially to develop the deep intracerebral microelectrode recording capability, which we estimate will require up to 10 animals. The actual study will use 10 rats/group: [(10 rats/group) x (3 groups: MitoQ/TPMP, MitoQ/vehicle, TPMP/vehicle) x 1.15 c.f.] + (10 rats for pilot study) = 45 rats. END OF WHOLE ANIMAL RAT EXPERIMENTS BEGIN RAT BRAIN SLICE EXPERIMENTS EXPERIMENT 4) Determine the effects of MitoQ during exposure to hyperoxia on the rate of production of reactive oxygen and nitrogen species and mitochondrial membrane potential in brain stem neurons in tissue slices. Brain slices are harvested from rats ranging in age from postnatal day 1 through 21 (P1-P21), weanlings, and young juveniles to young adults. All rats older are euthanized by rapid decapitation without CO2 anesthesia (we've reported that CO2 anesthesia alters reactive species production in brain stem during exposure to hyperoxia and carbon dioxide). For brain slice experiments to measure reactive species and mitochondrial membrane potential we estimate the following numbers of rats: Assumptions and power analyses used to determine the number of cells needed and animals needed for fluorescence imaging are explained below in Section 9. To measure reactive species: [(40 cells/treatment) x (6 rats/40 cells) x (2 dyes) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 40 rats; and to measure mitochondrial membrane potential: [(40 cells/treatment) x (6 rats/40 cells) x (1 dye) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 20 rats EXPERIMENT 5) Determine the effects of MitoQ on cellular O2-sensitivity using electrophysiology in rat brain slices. Brain slices are harvested from rats as in experiment 4. Estimated numbers of animals required are calculated as follows: [(10 slices/treatment) x (1 rat/cell) x (2 treatments: hyperoxia, MitoQ) x (1.4 c.f. x 2.2 c.f.) x (1 CNS nucleus)] = 62 rats In Section 9, we explain allowances for rat pups born but not used in experiments (13 rats, neonates & weaned rats born but not used in this research (too old for certain experiments)) and the damns required for pups (12 rats, timed-pregnant or nursing dams). In all, 135 rat pups need to be born to yield 40 + 20 + 62 = 122 rat pups for experiments. END OF RAT BRAIN SLICE EXPERIMENTS BEGIN AQUAPORIN-4 (AQP4) MOUSE EXPERIMENTS (no MitoQ or TPMP will be used in these experiments, only HBO) AQP4-KO and WT control mice will be shipped to USF Tampa from Case Western Reserve University, Cleveland, Ohio, Dept. of Physiology and Biophysics. Mice will initially be held in quarantine for 28 days in the COM vivarium before they are used in any experiments. CWRU will have a subcontract with USF on the USF Grant from ONR. EXPERIMENT 6) Effects of HBO on the risk for developing HBO-induced seizures in AQP4-KO vs. WT mice. After quarantine and acclimation to their new holding room, and being housed inside the animal & dive chambers, members of the Dean Lab will subject individual WT vs. AQP4-KO mice to HBO as in Experiment 2, above (rat chow & hydrogel are provided as in Expt. 2). Likewise, as in Experiment 2, no radio-telemetry is used. Visible seizures are scored using the behavior criteria. Each mouse will be dived four times to the point of 1st and 2nd bouts of visible seizures, one dive/week, over a 21-day period; e.g., 1st Monday (day 1), 2nd Monday (day 7), 3rd Monday (day 14), 4th Monday (day 21). Mice are returned to the COM vivarium between dives. THE RATIONALE FOR MULTIPLE DIVES AND SEIZURES is to induce kindling in mice and determine the effect of AQP4 on kindling. The number of mice required for this experiments is: (10 mice/group) x (2 groups: AQP4-KO & WT) = 20 mice total (10 AQP4-KO and 10 WT) EXPERIMENT 7) Effects of HBO -/+ CO2 retention on the risk for developing HBO-induced seizures in AQP4-KO vs. WT mice. As in Experiment 6 only now each mouse is dived twice, at least 1 wk apart, to the point of 1st and 2nd bouts of seizures (rat chow & hydrogel are provided as in Expt. 2). The order of HBO without CO2 and HBO with CO2 will be randomized between mice. Mice are returned to the COM vivarium between dives. The number of mice required for this experiments is: (10 mice/group) x (2 groups: AQP4-KO & WT) = 20 mice total (10 AQP4-KO and 10 WT) EXPERIMENT 8) Effects of hyperbaric oxidative preconditioning on AQP4 expression and the risk for developing HBO-induced seizures in WT mice. Prior to being dived to 5 ATA HBO, each mouse is pretreated (preconditioned) with a sublethal level of HBO (non-seizure promoting: 2.5 ATA HBO x 90-min) four times, interspersed by a 4.5 h room air breathing break, over a 24h period. Two mice are preconditioned at a time with access to food and hydrogel. All animal exposures are done in a Sechrist Animal Research Chamber (Model 1300B, MWP = 35 psig). The pressure chamber is ventilated continuously with air supplied from a compressed cylinder during the 4.5 h interval between 90-min

exposures to 2.5 ATA HBO. THE RATIONALE FOR HBO PRECONDITIONING DURING 24 HOUR PRIOR TO DIVING IS TO UPREGULATE AQP4 EXPRESSION IN THE BRAIN. After the 4th 90-min exposure to 2.5 ATA HBO, the mouse is kept on 1 ATA air for 4.5 h inside the preconditioning chamber. After that, the mouse is transferred to dive chamber (in the same room) and held on 1 ATA air (15min); Hydrogel and food pellets are placed on the floor of the animal chamber. Next, the ventilating gas is changed to 1 ATA O2 (15min) at which time the mouse is dived to 5 ATA HBO until the onset of 1st and 2nd bouts of seizures. After the 2nd bout of seizures, the mouse is decompressed to room pressure on air and anesthetized using isoflurane. The mouse remains on HBO up to 2 h or until onset of 2nd bout of seizures, which ever comes first. The total time spent inside the dive chamber is 2.5 h. A cardiac puncture (under anesthesia) is taken to measure the redox status of the animal using the redoxSYS analyzer (40uL blood/sample) and the perfused transcardially with buffer followed by paraformaldehyde fixative for immunohistochemical analyses of AQP4 expression in the brain. Three groups of WT mice will be studied; 1) mice preconditioned using 2.5 ATA HBO; control WT mice preconditioned with normoxic hyperbaric pressure (14% O2 + balance Helium = 0.21 ATA O2 + 0.79 ATA He), and another group of control WT mice maintained in the preconditioning chamber for 24h with ventilation using 1 ATA air. For example, the experimental protocol would be to initiate preconditioning at 1) 8am-9:30am, 2) 2pm-3:30pm, 3) 8pm-9:30pm, 4) 2am-3:30am, and then dive in HBO at 8am on next day. A member of the lab team will remain in the lab with the animal during the entire 24 h preconditioning period. The number of mice needed for this experiment is: (3 groups of preconditioned WT mice: HBO, normoxic hyperbaric pressure, and 1 ATA air) x (10 mice/group) = 30 WT mice

8.1.2 For complicated experimental designs, upload a detailed flowchart, diagram, or table, which depicts the experiments or sequence of events with a timeline, and indicates the number of animals needed for each experimental group and the number of groups required: >>Get Help

Name	Modified	Version
8.1.1 text with spacing for ease of reading	11/21/2019 3:18 PM	0.01
Flowchart logic of expts 1-8	11/22/2019 10:01 AM	0.01

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Statistical Analysis and Rationale

9.1

The following species/strain/lines were requested in Section 5. Use this table to complete your response to the questions below: >>Get Help

Species / Stock/Strain / Characteristics	# Pain Category A	# Pain Category B	# Pain Category C	Total Number
Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male)	0	0	50	50
Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)	0	0	305	305

Species / Stock/Strain / Characteristics	# Pain Category A	# Pain Category B	# Pain Category C	Total Number
Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)	0	0	20	20
Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)	122	0	0	122
Rat: Sprague-Dawley (neonates & weaned rats born but not used in this research (too old for certain experiments) but made available to other researchers on different protocols or euthanatized if unwanted)	13	0	0	13
Rat: Sprague-Dawley (timed-pregnant or nursing dams)	12	0	0	12

9.1.1 * **Describe the statistical analyses and rationale used to determine experimental group sizes and the total number of each species of animals requested:** >>Get Help

ANIMAL NUMBERS FOR PROJECT

WHOLE ANIMAL RAT EXPERIMENTS 1-3:

Part 1, Expt. 1) Up-and-Down Acute Toxicity Testing in rats to determine optimum dose of MitoQ to give via i.v., nas. & i.m. routes: (Note: no power analysis is required in this type of experiment) In brief, a single rat will be dosed by one of the three parenteral routes and observed for 2 days to determine the next dose; that is, the dose is increased if they live for 2 days, but if they die then the dose is lowered. Typically, the correct dose for each route can be identified after testing only 6-10 animals per delivery route. Each animal that survives the first two days will be observed for up to 7 days to establish any delayed onset toxicity to MitoQ. Toxicity will be assessed by monitoring animal activity 2-3 times/day for days 1-2 and then 1-2 times/day for days 3-7. Any animal that exhibits evidence of pain and discomfort will be removed and euthanatized; e.g., failure to respond to stimuli, inactivity, hunched or abnormal posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, seizures, vocalization, head pressing, and/or rapid/shallow/labored breathing. According to the literature, typically only 6-10 rats/delivery route will be needed to determine the safe vs. toxic dose of MitoQ; that is: [(3 routes: i.v., nas., i.m.) x (1 MitoQ) x (10 rats/route)] = 30 rats max.

Part 2, Expt. 1) Liquid Chromatography-Dual Mass Spectroscopy (LC/MS/MS) analyses of levels of MitoQ in the brain, blood and lungs in treated rats. MitoQ levels will be analyzed as a function of route of delivery (i.v., nas., i.m.) and time after dosing (0, 0.5, 1, 2 & 4 hr) using ANOV and multiple comparison testing ($P<0.05$). We have never done this type of experiment before; however, based on a previous LC/MS/MS study (Rodriguez-Cuenca et al., 2010, Consequences of long-term oral administration of the mitochondria-targeted antioxidant MitoQ to wild-type mice. Free Radical Biology & Medicine 48: 161-172), we plan to use six animals per group: [(6 rats/group) x (3 groups: i.v., nas., i.m.) x (1 substance: MitoQ) x (4 time points: 0.5, 1, 2 & 4 hr post-MitoQ injection)] + (6 rats: baseline group, time 0 (no MitoQ injection)) = 78 rats, where each animal donates brain stem, hippocampi, cerebral cortex, blood & lung tissues. (Also, in our past pharmacokinetic studies of blood levels of ketone ester at different time points post-dosing, we established significant differences in ketone body levels using 6 rats/group).

Experiment 2) Effect of HBO -/+MitoQ on latency time to onset of seizures in freely behaving rats. Based on our past studies of exposing rats to HBO, power analysis indicated a power = 0.8-0.9 for n=10 rats per group. Thus, for this experiment, we are requesting: [(10 rats/group) x (3 groups: MitoQ, TPMP, vehicle)] = 30 rats

Experiment 3) Telemetric Deep intracerebral microelectrode recordings from different brain regions in unrestrained, freely behaving rats during exposure to HBO -/+MitoQ. First, a pilot study will be done to establish the chronically indwelling telemetric intracranial microelectrode method in our lab. We estimate up to 10 rats will be required for this R&D phase of this experiment. Next, we recognize that failures (at 15% rate) may occur in radio-telemetry experiments that require removal of rats from the study due to one of the following problems: death during surgery, electrode failure post-surgery/during experimental sessions, radio-transmitter failure (battery failure), and unexpected illness/death during the study. Thus, a correction factor (c.f.) of 1.15 is used in animal numbers calculation to allow for 15% of animals that do not survive the experiment to completion for one of the above reasons. Based on our past telemetric experiments, power analysis indicated a power = 0.8-0.9 for n=10 rats per group. This is based on our previous experiments using radio telemetry to measure respiration, these procedures and the telemetric data generated. The actual study will use 10 rats/group: [(10 rats/group) x (3 groups: MitoQ/TPMP, MitoQ/vehicle, TPMP/vehicle) x 1.15 c.f.] + (10 rats for pilot study) = 45 rats.

RAT BRAIN SLICE EXPERIMENTS 4 & 5

Experiment 4) Fluorescence imaging in rat brain slices. To determine the number of cells needed and thus animals needed for proposed fluorescence imaging experiments (reactive species, mitochondrial membrane potential) are as follows: Power= 0.8/0.9, n=31/38 cells (std dev ~40 Fluorescence Intensity (FI) units/min, a = 0.05). Thus we will need ~40 cells/O2 protocol; 6-8 neurons/slice and 1 slice/rat (~6-8 neurons/rat and 5-6 rats to get ~40 cells, or say ~6 rats on average); and ~90% of rats will yield data (1/0.90=1.11 correction factor (c.f.) for ~10% of experiments failing to yield data). There are two fluorescent dyes used to measure superoxide and the aggregate pool of multiple reactive species. There are 3 test media.
[(40 cells/treatment) x (6 rats/40 cells) x (2 dyes) x (3 aCSF test media) x (1 CNS nucleus) x (1.11 c.f.)] = 40 rats for measuring reactive species in slices

Likewise, to measure mitochondrial membrane potential in slices: [(40 cells/treatment) x (6 rats/40 cells) x (1 dye) x (3 aCSF test media) x (1 CNS nucleus) x (1.11)] = 20 rats

Experiment 5) Electrophysiology in rat brain slices. Past experience (power analyses) have shown us that we need ~10 cells/ treatment to establish statistical significance for electrophysiological studies ($P<0.05$). Each cell serves as its own control. Differences in the means of firing rates, resting membrane potential and input resistances as a function of various test stimuli will be compared by two-way ANOVA with multiple paired comparisons done using Bonferroni's test ($P<0.05$). Assumptions used to determine the number of cells needed and animals needed are as follows: 1 useful cellular recording/rat and 80% of the experiments yield useful data (1/0.80=1.25 correction factor (c.f.) for rats that don't produce data); medullary neurons are heterogeneous in their O2- and CO2-sensitivity: only 50% of neurons tested are HBO-sensitive, and of these, 90% are CO2-excited. Thus, [(1/0.5) x (1/0.9)] = 2.2 correction factor (c.f.) to get adequate numbers of CO2- and O2-sensitive neurons to study; 2 treatments: hyperoxia; ~70% of experiments will last long enough to test O2 sensitivity -/+MitoQ at least once (1/.70=1.4, c.f.). Using these assumptions and facts, we estimate the maximum number of animals needed for electrophysiology experiments as follows:
[(10 slices/treatment) x (1 rat/cell) x (2 treatments x (1.4 x 2.2) x (1 brain nucleus)] = 62 rats

In addition, our estimates of animal numbers for neonates (Expts. 4 & 5) also takes into account animals that are born, but not used for brain slice experiments. Most of our experiments utilize neonatal rats, which typically are purchased weekly as i) a mother rat with newborn pups or ii) an untimed pregnant rat. Approximately 12 rat pups are born in each litter. We usually use ~90% of any given litter for our research. Any unused pups in the litter, which are subsequently weaned, are typically retained and used in experiments during the next month. If they are not needed, then weaned rats are transferred to another animal use protocol or disposed of in accordance with IACUC policies. However, any rat pups not used for slice experiments are also counted towards the total number of animals that are planned for use in this project. Thus, we

need to account for

(40 + 20 + 62)/0.90 = 122/0.9 = 135 rats born and 90% are used in slice experiments (i.e., 122 rats).

In addition, [(135 rats) x (1 litter/12 rats) = 12 dams per litter needed to produce 135 rats.

GRAND TOTAL OF RATS NEEDED FOR 3 YEARS: 305 + 122 + 13 + 12 = 452 Sprague-Dawley rats of various ages

AQUAPORIN KNOCKOUT MICE & WT CONTROLS

Experiment 6) Effects of HBO on the latency time to seizures in AQP4-KO mice vs. WT control mice. Based on our past studies of exposing rats to HBO, power analysis indicated a power = 0.8-0.9 for n=10 rats per group. We are assuming that this analysis extrapolates to our use of mice. The literature would suggest that is the case. Thus, for this experiment, we are requesting: [(10 mice/group) x (2 groups: AQP4-KO, WT mice)] = 20 mice (10 AQP4-KO, 10 WT).

Experiment 7) Effects of HBO -/+ carbon dioxide (CO_2) on the latency time to seizures in AQP4-KO vs. WT mice. As in Expt. 6, [(10 mice/group) x (2 groups: AQP4-KO, WT mice)] = 20 mice (10 AQP4-KO, 10 WT). Each mouse is dived twice and exposed to 1) HBO alone and 2) HBO + CO_2, order randomized between two dives.

Experiment 8) Effects of oxidative preconditioning on AQP4 expression and sensitivity to HBO -/+ CO_2. Three groups of WT mice will be tested in 5 ATA HBO: 1) WT mice preconditioned with 2.5 ATA HBO; WT mice preconditioned with 2.5 ATA normoxic Heliox; and WT mice "preconditioned" with 1 ATA air. Thus, as in Expt. 6, [(10 mice/group) x (3 groups)] = 30 WT mice.

9.1.2 * **Provide an explanation/rationale for assigning the specific number animals to the different pain categories of research declared above:**
Rats in pain category C (are there because they will experience potential distress from seizures caused by protracted exposure to HBO (153). Rats in pain category A are euthanized by rapid decapitation without anesthesia to harvest brain slices (122). Rats in pain category A are also there because they are either timed-pregnant or nursing dams (12) or rats born and not used for the proposed studies (13).
Mice in pain category C are there because they will experience potential distress from seizures caused by protracted exposure to HBO (80).

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Experimental Endpoints

10.1

10.1.1 * **Is animal death (excluding death from euthanasia) an <u>intentional</u> endpoint in this protocol?** >>Get Help

○ Yes ● **No**

10.1.1a **If Yes, explain why an earlier endpoint is not possible:**

10.1.2 * **Will animals be involved in procedures that are anticipated to produce more than momentary or slight pain, discomfort, or distress which cannot, or will not be alleviated by the administration of appropriate**

anesthetics or analgesics? >>Get Help

● **Yes** ○ No

10.1.2a **If Yes, describe the potential consequences of experimental manipulations, and the precise clinical criteria that will be used to ensure timely intervention:**

During HBO exposures, the animal (rats and mice) is visualized in real time continuously via video feed for telltale signs of CNS oxygen toxicity, including generalized tonic-clonic seizures of the upper limbs, neck and head coincident with increased brain wave activity (when measured). Once the 2nd bout of seizures begins (the 1st is minor and short lived lasting only a few seconds usually) the inspired [O2] is decreased and chamber decompression initiated. Typically, seizures stop immediately or within 1-2 minutes. If animals exhibit evidence of pain and discomfort at any time following surgery they will be removed and euthanatized; e.g., failure to respond to stimuli, inactivity, hunched or abnormal posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, seizures, vocalization, head pressing, and/or rapid/shallow/labored breathing.

10.1.2b **If Yes, describe the frequency of animal observation:**

During HBO exposures, the animal (rats and mice) is visualized in real time continuously via video feed for telltale signs of oxygen toxicity of brain (10.1.2a). All animals are checked daily while in the vivarium by a member of CompMed. Following surgery, animals are checked by a member of the P.I.'s lab for the next 1-3 days.

10.1.2c **If Yes, describe the intervention (e.g., treatment) used upon reaching the clinical endpoint, or indicate that animals reaching clinical endpoints will be euthanatized (but do not describe methods of euthanasia here, as these will be requested under item 11.1.1b):** >>Get Help

Any animal (rats and mice) exhibiting abnormal physiology or behavior will be removed and euthanatized with CO2.

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Euthanasia

11.1

11.1.1 * **Are animals euthanatized?**

● **Yes** ○ No

11.1.1a **If No, for each affected species, describe the final disposition of the animals:**

11.1.1b **If Yes, for each affected species, describe the method of euthanasia and means of ensuring death, indicating dose and route if a chemical agent or provide justification if a physical method:** >>Get Help

Rats in Experiments 1 (Parts 1 & 2), 2 & 3 are anesthetized by isoflurane and the euthanized by exsanguination.

Rats in Experiments 4 & 5 (brain slice harvesting) are euthantized by rapid decapitation without anesthesia. Rapid decapitation of fully awake animals will be conducted on an individual basis, and in a location away from other living animals. The decapitation instrument and the work bench where the procedure is performed will be thoroughly cleaned before bringing the next

animal into the area. Decapitation enables rapid isolation of the brain tissue with minimal physical trauma for brain slice preparation, which is critical for producing viable brain tissue slices for study. The animal box will be cleaned and dried between each use.

Dams and rat pups NOT USED in Expts 4 & 5 are placed in the CO2 gassing chamber (not precharged with CO2) and slowly filled with CO2 from a compressed gas tank. After cessation of breathing the animals are observed in room air for at least 10 minutes, or by employing a secondary method of euthanasia such as cervical dislocation, decapitation, or bilateral thoracotomy. The CO2 gassing chamber is cleaned and dried between each use. Alternatively, rats are transferred to another protocol. In all cases, decapitation without anesthesia will only be conducted by personnel whose verification of training has been documented by Comparative Medicine.

Mice in Expts 6-8 are euthanized using CO2 narcosis/asphyxia followed by a secondary method (cervical dislocation, rapid decapitation or bilateral thoracotomy).

Procedure Classifications

Indicate the procedures you will employ in this study from the list below.

* **Select all that apply:**

Section 13: **Husbandry & Caging:**

- [x] **Special Husbandry (Non-standard) or Housing at Satellite Locations**
- [x] **Food and/or Water Manipulation**
- [x] **Special Caging (Non-standard)**
- [ ] Exemption from Required Species-specific Enrichment
- [ ] Dog Exercise
- [ ] NHP Enrichment

Section 14: **Drugs Administered for Clinical Care:**

- [x] **Anesthetic, Analgesic, or Veterinary Therapeutic Administration (include drugs used during surgery)**

Section 15: **Test Substances Administered for Experimentation:** >>Get Help

- [ ] Biological Toxins/Infectious Agent
- [x] **Chemical Agent: Vehicle/Diluent/Solvent**

- [ ] Pharmacological Agent
- [ ] Radioactive Isotope
- [ ] Adjuvant/Antigen
- [ ] Tumor Cell Lines
- [ ] rDNA
- [ ] Primary Human Explants-Tissue, Cells, Fluids
- [ ] Other Agents

Section 16: **Specimen Collection:**

- [x] **Specimen Collection Ante mortem**
- [x] **Specimen Collection Post mortem (PI acknowledges that animals will be confirmed dead before tissues are collected.)**

Section 17: **Surgery:**

- [x] **Surgery (check Drug Administration above to account for drugs used during surgery)**

Section 18: **Other Experimental Procedures:**

- [ ] Irradiation
- [ ] Diagnostic Imaging & Microscopy
- [ ] Use of Human Patient Procedural Areas
- [ ] Breeding (Not Colony Production)
- [ ] Prolonged Restraint
- [ ] Stress Paradigms
- [ ] Behavioral Testing
- [x] **Other Experimental Procedure (Not listed above)**

Special Husbandry (Non-standard) or Housing at Satellite Locations

13.1.1 * **Name each affected species and describe all non-standard husbandry practices including the length of time implemented (e.g. modified diurnal cycle, enhanced immunocompromised rodent husbandry) or describe the maintenance/housing of animals for >12 hours at a satellite location outside of facilities:** >>Get Help

In Experiment 8, oxidative preconditioning, WT mice will be kept in one housing cage (2 mice/cage) inside the small hyperbaric chamber in (b) (6) for 24 hours. After 24 h, they are then dived in HBO in the larger chamber. After the dive in HBO, the two mice are either returned to the vivarium or euthanized.

13.1.2 * **Justify the implementation of this non-standard husbandry practice:**

The oxidative preconditioning protocol has been reported to upregulate expression of AQP4 in the mouse's blood-brain barrier. AQP4 is a water channel and a gas channel that is permeable to CO2 and presumably O2. The role, if any, of AQP4 in O2 and CO2 uptake during expsosure to HBO -/+CO2 is what we are trying to determine in these experiments. We predict that increased AQP4 expression will accelerate onset of CNS-OT seizures in WT mice vs. controls with lower level of AQP4 expression (normoxic Heliox, 1ATA groups).

13.1.3 **Proposals describing the maintenance/housing of animals for >12 hours outside of Comparative Medicine-managed facilities must upload a** Satellite Animal Facility Disaster Response Plan**.**

Name	Modified	Version
Satellite disaster plan for (b) (6)(b) (6)	11/21/2019 4:03 PM	0.01

Food and/or Water Manipulation

13.2

13.2.1 * **Name each affected species and describe all non-standard food and/or water manipulations (e.g. special dietary formulations, fluid supplements, food or water deprivation, calorie restrictions):** >>Get Help

During exposure to HBO in the pressure chambers, animals (rats and mice) will be provided food pellets on the floor of the animal chamber and hydrogel for fluid intake. Likewise, during 24h oxidative preconditioning the two mice will have unfettered access to food and hydrogel.

13.2.2 * **Justify the implementation of this non-standard food and/or water manipulation:**

The study requires the use of pressure chambers to induce oxidative preconditioning (24 hours) and HBO diving (2.5-3 hours) to study CNS-OT mitigation strategies.

13.2.3 * **Describe the methods used to monitor animal condition during the procedure and methods used to avoid and alleviate discomfort, if anticipated:**

During HBO dives, the animal is under constant digital camera and visual observation.

During the 24 hour of oxidative preconditioning, the animal is never left alone in

the room for more than 5 minutes (e.g., a bathroom break for the animal observer).

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Special Caging (non-standard)

13.3

13.3.1 * **Name each affected species and describe all non-standard caging used including the length of time housed (e.g. metabolic caging, wire mesh rodent caging, environmental chambers, other configurations):** >>Get Help

Sprague Dawley rat pups are used in many of the experiments. They are either purchased already born and with a nursing Dam or unborn in a gravid female. Each dam needs to be housed individually in its own cage with its litter.
For Expt. 8, WT mice, after quarantine, will need to be housed two/cage since that is how they will undergo oxidative preconditioning in (b) (6) .

13.3.2 * **Justify the implementation of this non-standard caging:**

Rats: This protects the neonatal rats from being injured by other adult rats.
WT mice: they will be exposed to oxdiative preconditioning two at time and then dived sequentially 24 hours later.

13.3.3 * **Describe the methods used to monitor animal condition during the procedure and methods used to avoid and alleviate discomfort, if anticipated:**

Rats and mice are checked daily by CompMed staff and frequently by members of the PI's lab.

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Drug Administration

14.1

14.1.1 * **Indicate the type(s) of drugs you will administer to animals during the course of this study.**

Anesthetics, analgesics and/or tranquilizers

Conventional Veterinary Therapeutics

14.1.2 * **Please press "Add" and complete the chart below for each drug used in this protocol. (Please note that you will be asked to provide further information for each drug on subsequent pages.)** >>Get Help

Drug	Pharmaceutical Grade?	Non Pharmaceutical Explanation	DEA Controlled?

	Drug	Pharmaceutical Grade?	Non Pharmaceutical Explanation	DEA Controlled?
View	isotonic saline	yes		no
View	Meloxicam	yes		yes
View	Isoflurane (Forane)	yes		no

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Anesthetics, Analgesics, Tranquilizers and Paralytics

14.2

14.2.1

*** Please press "Add" and complete the chart below for each drug used as an anesthetic, analgesic, tranquilizer, paralytic or reversal agent. (Do not include drugs used for therapeutic purposes):** >>Get Help

	Drug	Species	Dose	Route	Administration Frequency	Treatment Duration	Monitoring Efficacy
View	Isoflurane (Forane)	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)	3-5% induction; 1-3% maintenance	calibrated vaporizer, in O2, inhaled via nosecone; a waster anesthetic scavenging apparatus will be used in the plumbing circuitry	continuously until surgery ends	0.5-1.5 hrs	OLDER RATS (P11-21 and weaned) The level of anesthesia is determined by continuous monitoring of reflexes (Level of anesthesia will be assessed by interdigital/toe pinch, as well as the palpebral/eyeblink reflex. rate of breathing). If the animal becomes light during surgery (responds to interdigital toe pinch, etc.), then the level of isoflurane is raised by 0.5-1%.

	Drug	Species	Dose	Route	Administration Frequency	Treatment Duration	Monitoring Efficacy
View	Meloxicam	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)	1-2 mg/kg	s.c.	preemptive & post-op, 1x/day	injection	Meloxicam will be given as a preemptive analgesic in sterile saline prior to initiating surgery at any site. It is administered 1x/day and then p.r.n. at 24 hr intervals until 48 hours. Analgesic efficacy will be monitored post surgery by watching for signs of pain and discomfort; e.g., inactivity, hunched or abnormal posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, vocalization, head pressing, and/or rapid/shallow/labored breathing.

Conventional Veterinary Therapeutics

14.4

14.4.1 * Please press "Add" and complete the chart below for each therapeutic drug used: >>Get Help

	Drug	Species	Dose	Route	Administration Frequency	Treatment Duration	Monitoring Efficacy
View	isotonic saline	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)	10 ml/kg/hr	sc	3-5x boluses over duration of surgery	15-30sec/treatment	to rehydrate animal during surgery as needed

Chemical Agents - Vehicle - Diluent - Solvent

15.2

15.2.1 * **Indicate the type of Chemical Agent you will administer to animals in this study:**

- [x] **Conventional Chemical Name**
- [ ] Novel Chemical Name or Structure
- [x] **Vehicle/Diluent/Solvent**

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Conventional Chemical Details

15.3

15.3.1 * **Please press "Add" and complete the chart below for each Conventional Chemical used.** >>Get Help

View

Agent	Mitoquinone (MitoQ)
Species	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)
Dose/Volume/Route	TBD by up-and-down acute toxicity testing and LC/MS/MS; based on literature for mice we'll start at 20 mg/kg (750 nM); however, 27 mg/kg (1,000 nM) is toxic in mice / route TBD: i.v., nas. or i.m.
Frequency/Interval	1x
Duration	momentary
Pharmaceutical Grade?	Yes
Non Pharmaceutical Explanation	*No Value Entered*
Purpose of Substance	MitoQ is a supplement sold for human consumption. It is a mitochondria-targeted antioxidant.
Expected Effect	No adverse side effects are anticipated after up-and-down acute toxicity testing. Toxicity in mice, when reported, was not characterized other than "toxic".

View

Agent	Hyperbaric oxygen (HBO)
Species	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male) Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male) Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)
Dose/Volume/Route	2.5 and 5 ATA O2
Frequency/Interval	1-4x
Duration	from 90min to 2 hours maximum
Pharmaceutical Grade?	Yes
Non Pharmaceutical Explanation	*No Value Entered*
Purpose of Substance	HBO is used to induce CNS-OT seizures in rats and mice. We are studying the effects of MitoQ (rats) and AQP4 gas channels (mice) on the risk for developing CNS-OT while breathing HBO.
Expected Effect	HBO induces non-convulsive S/Sx that ultimately lead to tonic-clonic seizures. Non-convulsive S/Sx including bradycardia followed by tachycardia and hypertension and hyperventilation. Onset of seizures induces cardiogenic pulmonary edema. Lowering inspired oxygen will causes seizures to seizure and other physiological changes will reverse with varying time courses from minutes (cardiorespiration) to days (pulmonary edema).

View

Agent	triphenylphosphonium cation (TPMP)
Species	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)
Dose/Volume/Route	TBD by up-and-down acute toxicity testing and LC/MS/MS; based on literature for mice we'll start at 20 mg/kg (750 nM); however, 27 mg/kg (1,000 nM) is toxic in mice / route TBD: i.v., nas. or i.m.
Frequency/Interval	1x
Duration	momentary
Pharmaceutical Grade?	Yes
Non Pharmaceutical Explanation	*No Value Entered*
Purpose of Substance	TPMP is the molecule that is covalently bonded to the antioxidant coenzymeQ to produce MitoQ and make it permeable to the cell and mitochondrial membrane.
Expected Effect	No adverse side effects are anticipated after up-and-down acute toxicity testing. Toxicity in mice, when reported, was not characterized other than "toxic". TPMP and MitoQ are usually given in similar doses in animal experiments. TPMP is used as a control for MitoQ.

View

Agent	4.5 ATA O2 plus 0.5 ATA CO2
Species	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male) Mouse: Aquaporin-4 knockout (AQP4-KO) (2-3 mos./20-25g/male)
Dose/Volume/Route	4.5 ATA O2 plus 0.5 ATA CO2/continuous/inspired
Frequency/Interval	1-2x
Duration	up to 2 hours maximum
Pharmaceutical Grade?	Yes
Non Pharmaceutical Explanation	*No Value Entered*
Purpose of Substance	CO2 is added to the inspired O2 because hypercapnia induces cerebral vasodilation and accelerates onset of CNS-OT seizures. Also, AQP4 is permeable to CO2 and we want to determine if the onset of seizures is slowed in AQP4-KO mice due to slower uptake of CO2 during hypercapnic hyperoxia.
Expected Effect	CO2 will stimulate cardiorespiration and accelerate onset of CNS-OT seizures.

Vehicle/Diluent/Solvent Details

15.5.1 * Please press "Add" and complete the chart below for each Vehicle/Diluent/Solvent used. >>Get Help

View

Agent	soybean oil
Species	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)
Dose/Volume/Route	TBD by up-and-down acute toxicity testing and LC/MS/MS/ route = i.v., nas., i.m.
Frequency/Interval	1x
Duration	momentary
Pharmaceutical Grade?	No
Non Pharmaceutical Explanation	unavailable; soybean oil will be filtered prior to using
Purpose of Substance	the vehicle to be used for preparing MitoQ and TPMP for dosing by i.v., nas., i.m. While MitoQ is water soluble, TPMP is not.
Expected Effect	none expected

15.13.1 * **Is there a possibility that any of the test substance(s) could cause more than momentary or slight pain, discomfort, or distress to the animals, either immediately following substance administration, or as a consequence long after administering the test substance(s)?**

● Yes ○ No

15.13.1a **If Yes, for each such substance, describe the consequences of administration that have a potential to cause animal discomfort, pain, or distress, and how discomfort, pain or distress will be anticipated, minimized or alleviated (e.g., nude mice administered tumor cells will be humanely euthanatized if induced tumors become >1.0 cm in diameter, or if tumors ulcerate, or if tumors interfere with posture, locomotion or feeding). If discomfort, pain, or distress will not be alleviated, then justify how treatments interfere with the procedures or the interpretation of results:**

HBO will stimulate breathing and cause bradycardia initially. Without mitigation (MitoQ ?), the rat will seize, on average, after 30 min (5 ATA O2). With MitoQ, rat's latency to seizure may increase at 5 ATA O2. In this case, some animals may develop signs of pulmonary oxygen toxicity as evidenced by frothy fluid accumulation around oronasal region. In these cases, we will terminate the dive (we have seen this using ketone ester, which is another mitigation strategy used to delay seizures in HBO). Regarding MitoQ and TPMP, toxicity for these compounds has been reported in rodents but no specifics were mentioned regarding the characteristics of systemic toxicity other than stating that the compounds were "toxic".

15.13.2 * **Are any of the test substances listed above considered a regulated or potentially hazardous material to research or animal care personnel?**

○ Yes ● No

15.13.2a **If Yes, (1) list those substances in the space below, (2) describe procedures to ensure these substances and/or animals to which these substances have been administered will be handled safely (e.g., BSL-2, USF Chemical Hygiene Plan, Universal Precautions), and (3) provide an assurance that laboratory and animal care management and staff will be notified when such substances are in use:** >>Get Help

15.13.2b **Please upload any additional information regarding substances to be administered to animals (e.g., characterization of rodent biologics as pathogen-free, a detailed chemical structure for unique test substances that will allow their analysis for potential occupational hazards):**

Name	Modified	Version
There are no items to display		

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Specimen Collection *Ante Mortem*

16.1

16.1.1 * **For each specimen collected ante mortem (i.e., collections prior to euthanasia), press "Add" and complete the form.**

	Species	Tissue or Fluid Collected	Amount collected	Frequency	Method of Collection
View	Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)	arterial blood	100 uL	1x	cardiac puncture under anesthesia
View	Mouse: Wild Type C57/BL6 (2-3 mos./20-25g/male)	arterial blood	100 uL	1x	cardiac puncture under anesthesia

16.1.2 For specimen collection procedures that DO NOT have the potential of causing more than momentary pain or discomfort, describe the method of restraint and the method and site of collection: >>Get Help

In some experiments, rats or WT mice will be anesthetized using isoflurane and a cardiac puncture will be done to collect a blood sample to analyze the redox status of the animal. Only 40uL of blood are needed for the analysis. After harvesting the blood sample, the animal is euthanized by exsanguination for tissue harvesting alone or transcardial perfusion for immunohistochemistry and tissue harvesting.

16.1.3 For specimen collection procedures that DO have the potential of causing more than momentary pain or discomfort, describe the method of anesthesia including dose and route, the method of restraint and the method and site of collection: >>Get Help

The rat or mouse is anesthetized using isoflurane.

16.1.4 For specimen collections procedures that DO have the potential of causing more than momentary pain or discomfort, indicate whether post-procedural analgesics will be used, their dose, route, and frequency of administration, and if not used, a justification for their omission. Provide a description of the post-procedural methods of monitoring:

It is a terminal procedure; that is, after taking the blood sample, animal (rat or mouse) is euthanized.

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Surgery

17.1

Section 17 must be completed separately for each species used in surgical procedures. Select a species and all applicable strains/lines involved. If multiple species will be used in surgical procedures, answer "Yes" to 17.1.10 and complete an additional Surgery section.

17.1.1 * Species:

Rat: Sprague-Dawley (rats for MitoQ dosing, dives & telemetry) (5-12wk/150-350g/male)

17.1.2 * Select all that apply:

Survival

17.1.2a Please list personnel that will conduct survival surgical procedures. If delegated to Comparative Medicine surgeons, these surgeons need not be individually named: >>Get Help

Name	AALAS Aseptic Training	Aseptic Wet Lab Certificate	Link to Profile
(b) (6) (b) (6)	AALAS Rodent Aseptic Surgery (0.02)	Aseptic Surgical Training Wet Lab Cert(0.01)	(b) (6)
(b) (6) (b) (6)	AAALAS Certificate 12-11-2019.pdf(0.02)	trainingdocs.zip(0.01)	(b) (6)
PI: (b) (6) (b) (6) (b) (6)	ExamCertificate_aseptic surgery_(b) (6) (b) (6) (b) (6) pdf(0.01)		(b) (6)

17.1.3 * **Describe pre-operative medications, including pre-emptive analgesia, tranquilizers, sedatives, pre-anesthetics, and general anesthetics, and their dose and route:** >>Get Help

Note: the P.I. recognizes they need to take the wet lab hands-on training before they can participate in surgery to implant deep intracranial recording microelectrodes in rats.

No surgery is done on mice; only rats.

SURVIVAL SURGERY, DEEP INTRACRANIAL MICROELECTRODES & RADIO TRANSMITTER IMPLANT IN RATS. Each rat will undergo only one survival surgery to implant a 4ET telemetry transmitter. Sterile surgery will be conducted under general anesthesia. Rats will be anesthetized with isoflurane in an induction chamber using 3-5% isofluorane in O2 delivered via a scavenger-equipped calibrated vaporizer. After chamber induction of anesthesia, the rat is removed from the chamber and placed on a rodent-specific nose cone apparatus and maintained anesthetized using 1-3% with oxygen. Body temperature will be servocontrolled at 36-38degC using a heating pad and rectal temperature probe. A preemptive analgesic will be given (Meloxicam) in sterile saline prior to initiating surgery at any site. The level of anesthesia is determined by continuous monitoring of reflexes (eye blink, rate of breathing). If the animal becomes light during surgery (responds to tactile stimulation: eye blink), then an increase in % isoflurane be given until adequate anesthesia is achieved. Fluid replacement is given i.v. as needed.

17.1.4 * **Describe pre-operative procedures, including the withholding of food or water, (for what period of time), shaving of hair with enough border to prevent contamination of the operative site, three surgical germicidal scrubs of site, draping with sterile drapes, and the placement of leads, probes, or catheters.** >>Get Help

Food and water are NOT withheld before surgery.
For radio-transmitter implantation the abdominal, neck and head regions are clipped. All fur clipping is done away from the surgical table. The shaved site has enough border to prevent contamination of the operative site. The surgical sites are prepared by scrubbing thrice with the antiseptic chlorohexidine and rinsed with sterile saline. The surgical site is then draped with a sterile Bioclusive cloak. Surgical instruments to be used for surgery are autoclaved beforehand. The surgeon and assistant wear lab coats and sterile gloves during surgery. In multiple, same-day surgeries the surgical instruments are steam sterilized prior to use in the initial surgery. Prior to use the next surgery, surgical items are cleaned with sterile saline and sterilized using a hot-bead sterilizer or an alternate set of sterile instruments is used. If the radio telemetry transmitter is to be re-used we will follow the manufacturer's instructions for resterilization. The same sterilization process is used for the FHC tungsten electrode before it is trimmed to length using a pair of sterile scissors. Resterilization documentation is uploaded below: Actril Resterilization (392-0027-020).pdf

17.1.5 * **Describe each surgical procedure in chronological order and in sufficient detail so that the IACUC will be able to determine what is being performed on each animal:** >>Get Help

After the rat is anesthetized and the abdomen is opened to place the telemetry module and battery inside the animal. The abdomen is open for no

more than 10 to 15 minutes. The following procedures are done with no electrocautery necessary; i.e., the tissue dissection is not extensive and amounts to minimal and gentle displacement of organs for placement of telemetry module. 1) A sharp trocar is passed through the abdominal wall for passage of 4 pairs of wire leads through one opening. These leads will eventually be tunneled subcutaneously upwards along the back and neck to terminate at the opening in the skin over the skull. 2) The battery and telemetry module are inserted into the abdomen and the body cavity closed using absorbable sutures through the muscle wall. Absorbable sutures material suitable for internal use in mammals include polydioxanone (PDS®), polybutester (Novafil®), polypropylene (Surgilene®, Prolene®), and polygalactin 910 (Vicryl®). 3) An opening is cut in the skin over the skull and then the wire leads for the telemetry module are tunneled upwards subcutaneously to the opening in the skin over the skull. 4) The rat is secured in a stereotaxic frame using non-perforating ear bars. 5) Holes are drilled in the skull at up to four positions defined by coordinates given in the rat stereotaxic atlas for the brain nucleus targeted for recording microelectrode tip placement. Each tungsten microelectrode is attached to its telemetry lead as instructed in Papazoglou et al. 2016, J. Vis. Exp. (112): 54216. The electrode is cut to the required length and positioned stereotaxically using the micromanipulator. Likewise, the reference electrodes for each lead are positioned epidurally over the cerebellar surface. 6) Once all four recording and reference electrodes are positioned, they are fixed in place using dental epoxy. 7) The skin covering the skull is closed by nonabsorbable suture. 8) The animal is removed from the stereotaxic frame and placed in dorsal recumbency and the abdominal skin closed with wound clips, which are removed a week later. The following is a general synopsis of the sequence of surgical procedures. Typically, within 30 minutes of ending the surgery the rat will eat Cheerios when offered. A new trainee can do this surgery in no more than 2 hrs and the experienced surgeon routinely does this in 1 hr.

17.1.6 * **Describe pre- and intra-operative monitoring procedures of depth of anesthesia (e.g., rate & depth of respiration, inter-digital pinch or palpebral blink reflex), and describe all intra-operative medications and/or support:** >>Get Help

Isoflurane at 3-5% is used for induction of anesthesia. 1-3% in air or O2 will be used throughout all the surgical procedures. The level of anesthesia is determined by continuous monitoring of rate of breathing and periodic checks (every few minutes) of reflexes (toe & tail pinch, eye blink). If the animal becomes light during surgery (responds to toe & tail pinch, etc.) then an increase in %isoflurane will be given until adequate anesthesia is achieved.

17.1.7 * **Describe immediate post-operative monitoring procedures. Animals recovering from general anesthesia must be monitored at least until they are sternally recumbent and capable of purposeful movement.**

The animal will be watched until it is fully recovered. Rats will be assessed visually for respiratory rate and depth. Body temperature will be supported with a heating pad placed under the cage in a manner that allows the animal to move away from the heat source as they recover. Animals will be monitored continuously until they are able to exhibit purposeful movement and maintain sternal recumbency.

17.1.8 * **Describe post-operative monitoring and care after animals are returned to housing, including the administration of analgesics, medications, fluids, and any other support methods (dose, route and frequency of post-operative analgesics and medications must be described), and indicate that skin sutures or staples will be removed at approximately 10 - 14 days post-operatively:** >>Get Help

Survival for at least 7-10 days in the COM vivarium will be required to allow recovery from surgery (telemetry unit & intracranial microelectrodes implantation) so the animal can be studied in the awake state. Post-operative Meloxicam will be given for 24 hrs as described in 14.2.1. Until the day of the experiment, each operated rat will be checked daily by a member the P.I.'s lab or CompMed staff. As already stated, an animal exhibiting abnormal physiology or behavior will be removed and euthanatized with CO2; e.g., failure to respond to stimuli, inactivity, hunched or abnormal

posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, seizures, vocalization, head pressing, and/or rapid/shallow/labored breathing. Sutures will be removed by day 10 if animal has not been exposed to HBO during days 7-10.

17.1.9 * **Will more than one major survival surgical procedure be performed on a single animal? (Respond "No" if a survival surgical procedure is followed by a non-survival surgical procedure.)** >>Get Help

○ Yes ● No

17.1.9a **If Yes, name the procedures and provide scientific rationale/justification:**

17.1.10

For complicated surgical procedures/design/equipment etc., please upload a detailed diagram or SOPs:

Name	Modified	Version
Actril Resterilization (392-0027-020).pdf	11/8/2019 4:34 PM	0.01

17.1.11 * **Within this protocol, will another animal species be used in surgical procedures?**

○ Yes ● No

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Other Experimental Procedures

18.8

18.8.1 * **Please press Add and provide details for each other experimental procedure used:** >>Get Help

View

Species	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)
Name of procedure:	tattoo on tail
Describe the procedure:	Before a rat recovers from survival surgery, an identifying number will be tattooed on its tail using equipment borrowed from CompMed. This is so the animal's surgical injection of retrograde marker beads can be tracked when it is used to make brain slices from 15 hours to 14 days later.
How long will the procedure last?	<1min
Methods to minimize pain:	the animal will be anesthetized
Describe monitoring:	same as those descr bed for its survival surgery
Describe post-procedure monitoring:	same as those descr bed for its survival surgery
Will a log of observations be kept?	Yes

Species	Rat: Sprague-Dawley (neonates & weaned rats used in research to harvest brain slices/M&F)
Name of procedure:	up-and-down acute toxicity testing
Describe the procedure:	The up-and-down acute toxicity method will be used beginning with the dose recommended in mouse experiments; that is, a single dose of 20 mg MitoQ/kg body weight (0.75 mM MitoQ), which was reported to be "well tolerated" in mice; however, 27 mg/kg (1,000 nM) was found to be toxic to mice. In brief, a single unanesthetized rat will be dosed by one of the three parenteral routes (i.v., nas., i.m.) and observed for 2 days to determine the next dose; that is, the dose is increased if they live for 2 days, but if they die then the dose is lowered. Typically, the correct dose for each route can be identified after testing only 6-10 animals per delivery route.
How long will the procedure last?	Momentarily for the single injection of MitoQ by i.v., nas., and i.m. routes; however, the animal is returned to the vivarium and then monitored for the next 2 and 7 days. see: Lipn k R.L. et al. (1995) Comparison of the Up-and-Down, Conventional LD50, and Fixed Dose Acute Toxicity Procedures. Fd. Chem. Toxic. 33(3): 223-231; and Dixon (1991) Staircase Bioassay: The Up-and-Down Method. Neuroscience & Behavioral Reviews. 15: 47-50.
Methods to minimize pain:	The single injection i.v., nas., or i.m.
View **Describe monitoring:**	Each animal that survives the first two days will be observed for up to 7 days to establish any delayed onset toxicity to MitoQ. Toxicity will be assessed by monitoring animal activity 2-3 times/day for days 1-2 and then 1-2 times/day for days 3-7. Animal monitoring will be done the P.I.'s Lab personal, and their observations supplement those done daily by Comp Med staff. Each observation period done by the P.I.'s lab personnel will last at least 5 minutes. Any animal that exhibits evidence of pain and discomfort will be confirmed by consultation with the veterinary medical staff. The affected animal will be removed and euthanized; e.g., failure to respond to stimuli, inactivity, hunched or abnormal posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, seizures, vocalization, head pressing, and/or rapid/shallow/labored breathing.
Describe post-procedure monitoring:	Each animal that survives the first two days will be observed for up to 7 days to establish any delayed onset toxicity to MitoQ. Toxicity will be assessed by monitoring animal activity 2-3 times/day for days 1-2 and then 1-2 times/day for days 3-7. Animal monitoring will be done the P.I.'s Lab personal, and their observations supplement those done daily by Comp Med staff. Any animal that exhibits evidence of pain and discomfort will be removed and euthanized; e.g., failure to respond to stimuli, inactivity, hunched or abnormal posture, altered gait, ruffled/matted/or soiled fur, piloerection, circling, head tilt, seizures, vocalization, head pressing, and/or rapid/shallow/labored breathing.
Will a log of observations be kept?	Yes

Instructions for Submission

IS

You have reached the final page of this application.
Submission State: 18. Approved

If your submission state is "Pre Submission", please follow the steps below to submit this application for review.

1. Click the **Finish** button below to exit this form and return to the workspace.
2. Locate the **Submit for Veterinarian Review** activity on the left side of the workspace; click it, and follow any additional instructions to complete your submission.

Please Note: Following Veterinarian Review, the PI will be required to affirm the following upon submission to the IACUC:

- This description is complete and accurate.
- Written IACUC approval will be obtained prior to implementing changes to this protocol.
- Current biomedical supplies will be used.
- Complete animal procedural/surgical/testing records will be maintained.
- Conduct will be in accordance with the PHS Policy, AWR, The Guide, AAALAC Guidelines, DEA Regulations, and IACUC Principles and Procedures.
- Research personnel will abide by all relevant, universal precautions regarding blood-borne pathogens, appropriate biosafety level precautions, radiation safety procedures, and the chemical hygiene plan.
- All vertebrate animals or animal tissues will be acquired by lawful means, used judiciously, and disposed of appropriately.
- Personnel/students are adequately trained and experienced, or supervised to perform these procedures.
- Personnel/students are aware of occupational health & safety practices associated with the use of animals or animal tissues.
- IACUC applications describing teaching or training laboratories must assure the IACUC in writing that they or their designated IACUC-certified staff will continually directly supervise the students, and that prior to the lab commencing, will discuss with the students the potential risks and hazards associated with their involvement in the laboratory involving animals. All attending students will then complete a Student Safety in Teaching Laboratories Involving Animals form, which the PI will sign and provide to Comparative Medicine before the lab commences available at http://www.research.usf.edu/dric/iacuc/occupational-health-safety.asp

OMB Number: 4040-0001
Expiration Date: 10/31/2019

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE

State Application Identifier

1. TYPE OF SUBMISSION

☐ Pre-application ☒ Application ☐ Changed/Corrected Application

4. a. Federal Identifier N00014

b. Agency Routing Identifier 342 [redacted]

c. Previous Grants.gov Tracking ID

2. DATE SUBMITTED

Applicant Identifier

5. APPLICANT INFORMATION

Organizational DUNS: 0696872420000

Legal Name: University of South Florida

Department: Sponsored Research

Division: Research and Innovation

Street1: 4019 East Fowler Avenue Suite 100

Street2:

City: Tampa

County / Parish: Hillsborough

State: FL: Florida

Province:

Country: USA: UNITED STATES

ZIP / Postal Code: 336172008

Person to be contacted on matters involving this application

Prefix:

First Name: Brenda

Middle Name:

Last Name: Simmons

Suffix:

Position/Title: Sponsored Research Administrator

Street1: 4019 East Fowler Avenue Suite 100

Street2:

City: Tampa

County / Parish: Hillsborough

State: FL: Florida

Province:

Country: USA: UNITED STATES

ZIP / Postal Code: 336172008

Phone Number: 813-974-8778

Fax Number: 813-974-4962

Email: bsimmons@usf.edu

6. EMPLOYER IDENTIFICATION *(EIN) or (TIN)*: 59-3102112

7. TYPE OF APPLICANT: H: Public/State Controlled Institution of Higher Education

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. NAME OF FEDERAL AGENCY: Office of Naval Research

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER: 12.300

TITLE: Basic and Applied Scientific Research

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Electrodermal activity during hyberbaric oxygen exposure: rat study

12. PROPOSED PROJECT:

Start Date: 02/01/2019

Ending Date: 01/31/2020

13. CONGRESSIONAL DISTRICT OF APPLICANT

FL-014

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name: Jay Middle Name:
Last Name: Dean Suffix:
Position/Title: Professor
Organization Name: University of South Florida
Department: Molecular Pharm and Physiology Division: College of Medicine
Street1: (b) (6) [redacted]
Street2:
City: Tampa County / Parish: Hillsborough
State: FL: Florida Province:
Country: USA: UNITED STATES ZIP / Postal Code: 336129445
Phone Number: (b) (6) [redacted] Fax Number:
Email: (b) (6) [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	24,977.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	24,977.00
d. Estimated Program Income	0.00

16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

☒ **I agree**

**The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL (Disclosure of Lobbying Activities) or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: First Name: Keith Middle Name:
Last Name: Anderson Suffix:
Position/Title: Director
Organization: University of South Florida
Department: Sponsored Research Division: Research and Innovation
Street1: 4019 East Fowler Avenue Suite 100
Street2:
City: Tampa County / Parish: Hillsborough
State: FL: Florida Province:
Country: USA: UNITED STATES ZIP / Postal Code: 336172008
Phone Number: 813-974-2897 Fax Number: 813-974-4962
Email: rsch-awards@usf.edu

Signature of Authorized Representative	Date Signed
Raegen Einsmann	02/08/2019

20. Pre-application Add Attachment | Delete Attachment | View Attachment

21. Cover Letter Attachment Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0010
Expiration Date: 12/31/2019

Project Abstract

The Project Abstract must not exceed one page and must contain a summary of the proposed activity suitable for dissemination to the public. It should be a self-contained description of the project and should contain a statement of objectives and methods to be employed. It should be informative to other persons working in the same or related fields and insofar as possible understandable to a technically literate lay reader. This Abstract must not include any proprietary/confidential information.

* Please click the add attachment button to complete this entry.

Add Attachment | Delete Attachment | View Attachment

1234-ABSTRACT_Dean EDA CNS-OT study.p

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 0696872420000 **Enter name of Organization:** University of South Florida

Budget Type: ☒ Project ☐ Subaward/Consortium **Budget Period: 1** **Start Date:** 02/01/2019 **End Date:** 01/31/2020

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Jay		Dean		(b) (4)	0.24			(b) (4)	(b) (4)	(b) (4)

Project Role: PD/PI

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person (b) (4)

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Lab Manager - (b) (6)	1.08			(b) (4)	(b) (4)	(b) (4)
1	**Total Number Other Personnel**					**Total Other Personnel**	(b) (4)

Total Salary, Wages and Fringe Benefits (A+B) (b) (4)

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment Delete Attachment View Attachment

Total funds requested for all equipment listed in the attached file

Total Equipment

D. Travel

		Funds Requested ($)
1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2.	Foreign Travel Costs	
	Total Travel Cost	

E. Participant/Trainee Support Costs

		Funds Requested ($)
1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other	
	Number of Participants/Trainees — **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	820.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Telemetry (refurbish and batteries)	2,444.00
9.	Animal Costs	1,000.00
10.	GSR Amp for AD Instruments	1,170.00
	Total Other Direct Costs	5,434.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	16,707.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	(b) (4)	(b) (4)	(b) (4)
		Total Indirect Costs	(b) (4)

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

DHHS, Shon Turner, 214-767-3261

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	24,977.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	24,977.00

L. Budget Justification

(Only attach one file.) 1238-BUDGET JUSTIFICATION_Dean EDA CN$ Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		(b) (4)
Section B, Other Personnel		(b) (4)
Total Number Other Personnel	1	
Total Salary, Wages and Fringe Benefits (A+B)		11,273.00
Section C, Equipment		
Section D, Travel		
1. Domestic		
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		5,434.00
1. Materials and Supplies	820.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	2,444.00	
9. Other 2	1,000.00	
10. Other 3	1,170.00	
Section G, Direct Costs (A thru F)		16,707.00
Section H, Indirect Costs		(b) (4)
Section I, Total Direct and Indirect Costs (G + H)		24,977.00
Section J, Fee		
Section K, Total Costs and Fee (I + J)		24,977.00

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: * First Name: Jay Middle Name:
* Last Name: Dean Suffix:
Position/Title: Professor Department: Molecular Pharm and Physiology
Organization Name: University of South Florida Division: College of Medicine
* Street1: 12901 Bruce B Downs Blvd MDC8
Street2:
* City: Tampa County/ Parish: Hillsborough
* State: FL: Florida Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 336129445
* Phone Number: (b) (6) Fax Number:
* E-Mail: (b) (6)

Credential, e.g., agency login:

* **Project Role:** PD/PI **Other Project Role Category:**

Degree Type: PhD
Degree Year: 1986

*Attach Biographical Sketch 1237-Biosketch_Dean, Jay.pdf Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Department:
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

* **Project Role:** **Other Project Role Category:**

Degree Type:
Degree Year:

Attach Biographical Sketch Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 10/31/2019

1. Are Human Subjects Involved? ☐ Yes ☒ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☒ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number: A4100-01

3. Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract 1235-Project Abstract.pdf [Add Attachment] [Delete Attachment] [View Attachment]

8. Project Narrative 1236-Technical Proposal.pdf [Add Attachment] [Delete Attachment] [View Attachment]

9. Bibliography & References Cited [Add Attachment] [Delete Attachment] [View Attachment]

10. Facilities & Other Resources [Add Attachment] [Delete Attachment] [View Attachment]

11. Equipment [Add Attachment] [Delete Attachment] [View Attachment]

12. Other Attachments [Add Attachments] [Delete Attachments] [View Attachments]

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED PERSONAL DATA
Project Director/Principal Investigator and Co-Project Director(s)/Co-Principal Investigator(s)

The Federal Government has a continuing commitment to monitor the operation of its review and award processes to identify and address any inequities based on gender, race, ethnicity, or disability of its proposed PDs/PIs and co-PDs/PIs. To gather information needed for this important task, the applicant should submit the requested information for each identified PD/PI and co-PDs/PIs with each proposal. Submission of the requested information is voluntary and is not a precondition of award. However, information not submitted will seriously undermine the statistical validity, and therefore the usefulness, of information received from others. Any individual not wishing to submit some or all the information should check the box provided for this purpose. Upon receipt of the application, this form will be separated from the application. This form will not be duplicated, and it will not be a part of the review process. Data will be confidential.

Project Director/Principal Investigator

Prefix:

*** First Name:** Jay

Middle Name:

*** Last Name:** Dean

Suffix:

Gender: Male

Race (check all that apply):

- [ ] American Indian or Alaska Native
- [ ] Asian
- [ ] Black or African American
- [ ] Native Hawaiian or Other Pacific Islander
- [ ] White
- [x] Do Not Wish to Provide

Ethnicity: Non-Hispanic or Latino

Disability Status (check all that apply):

- [ ] Hearing
- [ ] Visual
- [ ] Mobility/Orthopedic Impairment
- [ ] Other
- [ ] None
- [x] Do Not Wish to Provide

Citizenship: US Citizen

Abstract

(b) (4)
[redacted] Aim 1 will be done at USF in the laboratory of co-I Dr. J. B. Dean using unanesthetized, instrumented adult male Sprague-Dawley rats. The goal of this aim is to evaluate the utility of measuring electrodermal activity (EDA) across the skin as a physiological marker of an impending grand mal tonic-clonic seizure, which the hallmark symptom of central nervous system oxygen toxicity (CNS-OT) while breathing hyperbaric oxygen (HBO_2). We hypothesize that increased EDA can be detected prior to onset of seizures while breathing HBO_2 and thus used to predict an impending episode of CNS-OT. Increased EDA has been observed prior to onset of epileptic seizures at room pressure in rodents. To accomplish this aim, rats are anesthetized and implanted with a radio telemetric module for measuring various physiological parameters. At least one week later, under anesthesia, two EDA electrodes are attached to the rodent's back using either surface electrode attachment or subdermal attachment. 24 hours later, the animal is placed in the animal chamber inside the hyperbaric chamber and the two EDA wire leads are connected to the animal. The EDA lead wires are restrained by a tether harness. The animal is dived to 5 ATA HBO_2 until onset of first and second bouts of seizures. The importance of this study is to determine if electrodermal activity, which can be easily monitored in animals and humans, is a useful physiological marker of an impending CNS-OT "hit"; that is, does EDA increase prior to seizure onset, and can it be used as signal for taking corrective action by lowering inspired oxygen and thus averting CNS-OT seizures.

Budget justification:

Key personnel—($11,273/yr 1)

Jay B. Dean (PhD; 2% effort) (b) (4) [redacted] Dr. Dean is director of the USF Hyperbaric Biomedical Research Laboratory and an expert in hyperbaric physiology and neurophysiology. Dr. Dean will take responsibility for all the proposed animal experiments in Aim 1, which measures EDA before and during onset of CNS-OT seizures in rats.

(b) (6) [redacted] (BS; 9% effort) is the lab manager for the USF Hyperbaric Biomedical Research Laboratory. (b) (6) [redacted] is involved in all animal experiments including surgery to implant the radio telemetry module and in assisting the Co-I in conducting and analyzing the experiments.

Materials and supplies—($820/yr 1) Funds are requested to purchase surgical supplies, anesthetics and analgesics, and gases that are used for implanting radio-telemetry modules and EDA leads under sterile conditions. Gases are also used for administering HBO to induce CNS-OT.

Other: telemetry ($2,444/yr 1), animal costs ($1,000/yr 1), and GSR Amp for ADInstruments data acquisition system ($1,170/yr 1)—Funds are requested to have Data Sciences International (DSI) refurbish four F-40 radio-telemetry modules that we currently have in the lab. Funds are also requested for 12 animals, which includes purchase price plus shipping and per diem costs. Finally, a GSR amplifier that interfaces with a preexisting ADInstruments data acquisition system is requested to measure EDA in rats.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Jay B. Dean, PhD	POSITION TITLE Professor of Molecular Pharmacology & Physiology
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Central Michigan University, Mt. Pleasant, MI	B.S.	8/79	Biology, Chemistry
Michigan Technological Univ., Houghton, MI	M.S.	5/81	Biol. Sci. (Respiratory Control)
The Ohio State University, Columbus, OH	Ph.D.	12/86	Physiology (Control of body temperature)
Univ. of North Carolina, Chapel Hill, NC	Post-Doc.	12/86-11/91	Neurophysiology (Resp.Control)

A. Personal Statement

My lab studies the effects of O_2, CO_2 and barometric pressure on the function of the mammalian CNS, with an emphasis on the cardio-respiratory and gastro-esophageal control systems. Ongoing research focuses on understanding how hyperoxia and hypercapnia interact to generate reactive O_2 and N_2 species that, in turn, activate neuronal activity in brain stem neurons. This research has relevance to understanding normal and abnormal function in cardio-respiratory control during periods of disordered breathing, environmental stresses, and in understanding neurological problems arising from the use of hyperoxia in clinical medicine and combat operations. We are particularly interested in the following: 1) neurophysiology of CNS oxygen toxicity (seizures) and its mitigation by ketone metabolic therapy; 2) understanding how CO_2 and H-ions are "sensed" by chemoreceptor neurons in the caudal medulla oblongata; and 3) understanding how gastro-esophageal CO_2 ventilation supplements pulmonary ventilation during acute and chronic respiratory acidosis, digestion and other conditions that stimulate gastric acid secretion.

B. Positions and Honors

Positions and Employment

1986-88	NINCDS Postdoctoral Fellow, Dept. of Physiology, Curriculum in Neurobiology, UNC-CH (D.E. Millhorn)
1988-90	NRSA Postdoctoral Fellow, Dept. of Physiology, UNC-CH (D.E. Millhorn)
1990-91	Res. Assist. Prof./Parker B. Francis Fellow in Pulmonary Res., Dept. of Physiology, UNC-CH (D.E. Millhorn)
12/91-97	Assistant Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
1997-02	Associate Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
7/02-6/03	Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
7/03-10/04	Acting-Chairman, Dept. of Anatomy & Physiology, Wright State Univ., Dayton, OH
11/04-3/05	Vice Chair of Administration, Dept. of Anatomy & Physiology, Wright State Univ., Dayton, OH
3/05-6/06	Vice Chair of Administration, Dept. of Neuroscience, Cell Biology and Physiology, WSU, Dayton, OH

7/1/06-pres. Professor, Dept. of Molecular Pharmacology & Physiology, Coll. of Med., Univ. of South Florida, Tampa, FL

7/1/10-10/30/11 Acting Chair, Molecular Pharmacology & Physiology, Coll. of Med., Univ. of South Florida, Tampa, FL

Other Experience and Professional Memberships

1992-94 NSF, Special Emphasis Panel for Research Planning Grants and Career Advancement Awards for Women

1998 NIH, Special Emphasis Panel

2000 NIH, Respiratory and Applied Physiology Study Section, Temporary Member

2002 NIH, Respiratory Physiology Study Section, Ad-hoc Member

3/2003 NIH grant review panel for SCOR grants, Sleep Apnea

10/03-6/07 Regular member, NIH Respiratory Integrative Biology and Translational Research Study Section (RIBT)

1999-00 Co-organizer & Lecturer, International Symp., Central Chemoreception 2000, Ruhr Univ., Bochum, Germany

2001-02 Co-chair & Lec., Featured Topic, EB2002, Role of gap junctions in CO_2-chemoreception & respiratory control

2000-01 Guest Co-Ed., Special Issue, Respir. Physiol., Central Chemoreception 2000 (vol. 129:1-278, 2001)

2003-04 Chair & Lecturer, Featured Topic, EB 2004, Oxygen Sensing by Neural Tissues

4/10-6/10 Co-Ed., Highlighted Topic, J. Appl. Physiol., Central CO_2 Chemoreception & Cardio-respiratory Control

1999-pres. Editorial Board, Journal of Applied Physiology

2008-2010 Associate Editor, Journal of Applied Physiology (Hyperbaric Physiology, Respiratory Control)

2002- 2012 Editorial Board, Undersea and Hyperbaric Medicine

1987- pres. Member, Society for Neuroscience

1997- pres. Member, American Physiological Society

1997- pres. Member, Undersea and Hyperbaric Medical Society

2000- pres. Member, Society for Free Radical Biology and Medicine

2004- pres. Member, Aerospace Medical Association

Honors

7/03-6/06 Brage Golding Distinguished Professor of Research, Wright State Univ., Dayton, OH

Selected peer-reviewed publications pertinent to this application (from a total of >60)

Ciarlone, G.E., Hinojo, C.M., Stavitzski, N.M., and Dean, J.B. (2019) CNS function and dysfunction during exposure to hyperbaric oxygen in operational and clinical settings. Invited review for Redox Biology, Special Issue entitled, "Redox Biology in Physiology and Disease" (in peer review; manuscript no. REDOX_2019_115)

Ciarlone, G.E., and Dean, J.B. (2019) Acute hyperoxia increases CO_2-chemosensitivity in rat caudal solitary complex neurons. American Journal of Physiology, Cell Physiology (in revision; manuscript C-00368-2018R1)

Ari, C., Koutnik, A. P., DeBlasi, J., Landon, C. S., Rogers, C. Q., Vallas, J., Bharwani, S., Puchowicz, M., Bederman, I., Diamond, D.M., Kindy, M.S., Dean, J.B., and D'Agostino, D. P. e. (2019). Delaying latency to hyperbaric oxygen-induced CNS oxygen toxicity seizures by combinations of exogenous ketone supplements. Physiological Reports. doi: 10.14814/phy2.13961

Morris K.F., Nuding S.C., Segers L.S., Iceman K.E., O'Connor R., Dean J.B., Ott M.M., Alencar P.A., Shuman D., Horton K.K., Taylor-Clark T.E., Bolser D.C., and Lindsey B.G. (2017) Carotid chemoreceptors tune breathing via multipath routing: Reticular chain and loop operations supported by parallel spike train correlations. J Neurophysiol 119(2):700-722. doi: 10.1152/jn.00630.2017

Ciarlone G. E. and Dean J. B. (2016a) Normobaric hyperoxia stimulates superoxide and nitric oxide production in the caudal solitary complex of rat brain slices. Am J Physiol Cell Physiol 311: C1014–C1026,

Ciarlone G. E. and Dean J. B. (2016b) Acute hypercapnic hyperoxia stimulates reactive species production in the caudal solitary complex of rat brain slices but does not induce oxidative stress. Am J Physiol Cell Physiol 311: C1027–C1039, 2016.

Gianni D'Angelo, Raffaele Pilla, Jay B. Dean, Salvatore Rampone (2016) Toward a soft computing-based correlation between oxygen toxicity seizures and hyperoxic hyperpnea. Soft Computing (METHODOLOGIES AND APPLICATION), DOI 10.1007/s00500-017-2512-z

Matott, M.P., Ciarlone, G.E., Putnam, R.W., and Dean, J.B. (2014) Normobaric hyperoxia (95% O_2) stimulates CO_2-sensitive and CO_2-insensitive neurons in the caudal solitary complex of rat medullary tissue slices maintained in 40% O_2. Neuroscience 270: 98-122

Held, H.E., Pilla, R., Ciarlone, G.E., Landon, C.S., Dean, J.B. (April 2014) Female rats are more susceptible to central nervous system oxygen toxicity than male rats. Physiological Reports 2 (e00282) DOI: 10.14814/phy2.282; http://physreports.physiology.org/content/2/4/e00282.short

Pilla, R., Held, H.E., Landon, C.S., and Dean, J.B. (2013) High doses of pseudoephedrine hydrochloride accelerate onset of CNS oxygen toxicity seizures in unanesthetized rats. Neuroscience 246: 391-396.

D'Agostino, D.P., Pilla, R., Held, H.E., Landon, C.S., Puchowicz, M., Brunengraber, H., Ari, C., Arnold, P. and Dean, J.B. (2013) Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. Am. J. Physiol. Reg., Integr. and Comp. Physiol. 304(10): R829-R836

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D. Research Support

Ongoing Research Support

ONR N00014-16-1-2537 Dean (PI) 6/1/2016 – 5/31/2019
(no cost extension to be requested: -8/31/2019)

Office of Naval Research, Undersea Medicine Program

Cellular mechanisms of CNS oxygen toxicity during CO_2 retention and ketone metabolic therapy

The goal of this research project is to further understand how conditions that accelerate and delay onset of CNS oxygen toxicity (CNS-OT) seizures (Sz) affect each of the following phenomena that are implicated in CNS-OT in rodents: i) increased production of reactive O_2 and N_2 species \ in the brain stem; ii) electrical signaling by medullary neurons; and iii) respiration during the period preceding Sz; i.e., hyperoxic hyperventilation. iv) An additional goal is to determine the effects of chronic CO_2 retention on cardiorespiratory and gastric physiology.

ONR N00014-18-1-2701 D'Agostino (PI) 08/01/2018 – 7/31/2021

Office of Naval Research

Optimizing ketone metabolic therapy and identifying biomarkers for mitigation and prediction of CNS oxygen toxicity: animal studies

The major goal of this project is to define the efficacy and anticonvulsant mechanism of ketones using in vivo and in vitro models

JBD's Role: Co-I

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Volume 1: Technical Proposal

BAA Number: ONR BAA Number: N00014-19-S-B001

Title of Proposal: Aim 1, Animal studies (USF Tampa): Feasibility of electrodermal activity for detecting seizures elicited by central nervous system oxygen toxicity

Prime Offeror: Office of Naval Research, Undersea Medicine Program

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• TECHNICAL APPROACH AND JUSTIFICATION[1]

1. Introduction

HBO_2 & CNS oxygen toxicity. Hyperbaric oxygen (HBO_2) is breathed during hyperbaric oxygen therapy (HBOT) for treating problematic wounds and resolving disorders caused by inert gas bubbles and emboli [1]. HBO_2 is also used for certain specialized undersea activities covered under recreational, commercial, and military diving operations [2, 3], and pressurized disabled submarine (DISSUB) emergencies that require escape or rescue [4-6]. In these contexts, the greatest benefit of using O_2-enriched gas mixtures or pure O_2 is the avoidance of maladies that involve inert gases [7-9]. Breathing a hyperoxic gas prior to decompression, a strategy known as oxygen prebreathing or isobaric denitrogenation, can be used to reduce or omit decompression obligations and avert the risk for decompression sickness (DCS) not only in divers completing prolonged dive profiles, but also in pressurized DISSUB personnel who are exposed to hyperbaric air inside a compromised submarine at a maximum rescuable depth equivalent of 5 ATA ambient pressure [4-6]. Importantly, high dose O_2 can also be used in conjunction with closed-circuit underwater breathing apparatus (e.g., MK-25 and LAR V Dräger rebreathers) in military clandestine operations to maintain stealth (no exhaled gas bubbles) during shallow water transit [3, 9, 10].

What limits exposure to HBO_2 in the foregoing situations is the risk of developing central nervous system oxygen toxicity (CNS-OT) following a latent period of safe oxygen breathing. CNS-OT presents as various non-convulsive signs and symptoms, many of which appear to be of brainstem origin involving cranial nerve nuclei, autonomic nervous system centers, and cardiorespiratory centers, which ultimately spread to higher cortical centers and terminate as grand mal tonic-clonic seizures [11]. The initial safe latent period makes the use of HBO_2 practical in hyperbaric and undersea medicine; however, the latent period is highly variable between individuals and within the same individual on different days, making it difficult to predict onset of lethal indications of CNS-OT. Consequently, currently accepted guidelines for safe HBO_2 exposure are highly conservative; for example, current guidelines for safe exposure at 50 feet of sea water (fsw) breathing pure oxygen is 10 minutes [12].

The purpose of this research project is to test the hypothesis that skin electrical resistance, which is known as electrodermal activity (EDA), increases during the pre-seizure period of CNS-OT. The utility of this approach is that increased EDA, if it precedes seizures sufficiently early, could be used to take corrective action and avert seizures; i.e., reduce the level of inspired partial pressure of oxygen (P_IO_2). Early changes in animal physiology that precede seizures during exposure to HBO_2 are known as **physiological markers** or **physio-markers** of an impending seizure [11]. In the proposed study, the animal's physiology will be measured using radio telemetry and EDA electrodes attached to the back of the animal while breathing HBO_2 in a

---

[1] ***Abbreviations used in grant proposal:*** **AFM,** atomic force microscopy; **ATA,** atmospheres absolute pressure; **CBF,** cerebral blood flow; **CNS,** central nervous system; **CNS-OT,** central nervous system oxygen toxicity; **DCS,** decompression sickness; **DISSUB,** disabled submarine; **EDA,** electrodermal activity; **EEG,** electroencephalogram; **F_IO_2,** fractional concentration of inspired oxygen; **fsw,** feet of sea water; **HBO_2,** hyperbaric oxygen; **HBOT,** hyperbaric oxygen therapy; **$NormO_2HBHe$,** normoxic hyperbaric helium (heliox); **He,** helium; **LSz,** latency time to seizure; **LSz1 & LSz2,** latency time to 1st and 2nd bouts of seizures; **O_2,** oxygen; **P_B,** barometric pressure; **PO_2,** partial pressure of oxygen; **P_IO_2,** partial pressure of inspired oxygen; **t_0,** time zero for calculation of LSz; **USN,** United States Navy

pressure chamber. Onset of CNS-OT will be determined by the appearance of neurological and behavioral (motor) seizures [13].

Logistics and personnel. (b) (4) [redacted] Aim 1 (the present study) will be done at USF in the laboratory of Dr. J.B. Dean using unanesthetized, instrumented adult male Sprague-Dawley rats. Experiments for Aim 1 will be done by the co-I. (J.B. Dean, 2% FTE) assisted by (b) (6) [redacted] (Lab Manager, 9% FTE).

2. Specific Aim

Aim 1. *Determine the effects of HBO_2 on electrodermal activity (EDA) in Sprague-Dawley rats*. Changes in EDA will be correlated with other physiological and behavioral parameters including cortical electroencephalogram activity, behavioral motor seizures, heart rate and pattern, respiration, body temperature, and animal activity.

3. Background

HBO_2 & physio-markers that precede seizures. Potential physio-markers under investigation for predicting an impending seizure during exposure to HBO_2 include the following: hyperoxic hyperpnea, which is also known as hyperoxic hyperventilation [14, 15]; heart rate and pattern [16, 17]; and hyperoxic hypothermia [18-20]. Several other possible predictors exist [3, 10, 21], most of which appear to be of brainstem origin [11]. The proposed study seeks to determine if increased EDA is another physio-marker of an impending CNS-OT seizure.

Electrodermal activity (EDA). EDA is a measure of the electrical activity of sympathetic (autonomic) nerves that innervate the skin [22-24]. Previously, it has been shown that EDA increases prior to onset of epileptic seizures in humans [22]. **The proposed study will test the hypothesis** that increased sympathetic neural activity precedes seizure genesis during HBO_2 exposure in a commonly used small animal model of CNS-OT; i.e., the adult male Sprague-Dawley rat [15, 25-28]. Good EDA signals can be measured from the back of a semi restrained (tethered), unanesthetized rat [24].

The pathophysiology of CNS-OT: role of increased autonomic neural activity during early exposure to HBO_2. Research, much of it supported by the ONR Undersea Medicine Program, has shown that exposure to HBO_2 initially results in a transient parasympathetic response comprised of decreased heart rate, cardiac output, blood pressure, and sympathetic tone [29]. Initially, cerebral blood flow (CBF) is decreased by vasoconstriction and delays a significant increase in brain tissue P_{O_2} [30-35]. Likewise, minute ventilation decreases initially due to hyperoxic inactivation of peripheral chemoreceptors [14, 15]. With extended exposure to HBO_2, this cardiopulmonary response is overtaken by increased sympathetic outflow resulting in hyperventilation, and hypertension [15, 29, 36]. We plan to determine if part of the increased sympathetic nervous system response includes an increase in EDA. In parallel with the sympathetic response, a buildup in nitric oxide causes escape from O_2-induced cerebral vasoconstriction and CBF increases causing a surge in neural tissue P_{O_2} and reactive O_2 and N_2 species production and, shortly thereafter, seizures [32, 37, 38].

Knowledge Gaps. This is the first study to determine if increased EDA precedes onset of CNS-OT in mammals. Changes in sympathetic EDA will be correlated with other physiological parameters including respiration, heart rate, core body temperature and the latency time to onset of neurological and behavioral seizures (LSz). If changes in EDA activity correlate with the LSz, then attempts will be made to determine if monitoring relative level of EDA can be used to avert CNS-OT seizures in our proposed animal model.

4. Specific Experiments

Aim 1. Determine the effects of HBO_2 on EDA in unanesthetized rats.

Hypothesis 1. Skin electrical resistance, which is known as EDA and a function of sympathetic neural activity, increases during the early pre-seizure period of CNS-OT and forewarns of an impending seizure.

Rationale 1. We know that the early pre-seizure period of exposure to HBO_2 is a period of increased autonomic nervous system activity that initially begins as parasympathetic activation that then switches over to increased sympathetic neural activity [11]. We also know that increased EDA precedes onset of epileptic seizures under normobaric (room pressure) normoxic conditions [22]. Furthermore, CNS-OT seizures are thought to resemble the seizures in idiopathic epilepsy [11]. Thus, we postulate that increased EDA activity precedes onset of HBO_2-induced convulsions.

Technical Approach 1.

Overview. Each rat will be implanted with a telemetric unit to measure cortical electroencephalograph (EEG) activity. Brain oxygen toxicity will be measured by onset of neurological seizures (increased EEG activity) and behavioral (visible tonic-clonic motor) seizures. Latencies to first and second bouts of seizures will be determined (LSz1 & LSz2). The first bout of seizures is usually small and short duration [13]. Typically, seizures stop immediately or within 1-2 minutes once P_{IO_2} is reduced. EDA will be measured by quantifying skin conductance changes due to changes in sympathetic nervous activity measured by either skin or sub-dermal electrodes. Initially, a small pilot experiment will determine the best method to measure EDA in rats during exposure to HBO_2 (5 ATA O_2); both skin electrodes and sub-dermal wire electrodes will be tested. Once this is established, each animal will be exposed to 5 ATA HBO_2 until the onset of the 2nd bout of seizures (LSz2). The animal will be decompressed on air and returned to the vivarium after it exhibits normal behavior. Five to seven days later, the same animal will be dived on normoxic heliox using 4%O_2 + 96% helium (He) to produce 0.2 ATA O_2 + 4.8 ATA He at total ambient pressure of 5 ATA; i.e., normoxic hyperbaric helium (NormO_2HBHe). An equivalent period of exposure to NormO_2HBHe serves as a control for the effects of pressure per se to confirm that any observed changes in EDA during HBO_2 are due to increased P_{O_2} and not due to hyperbaric pressure per se. The order of HBO_2 and NormO_2HBHe dives are not randomized because we need to know the duration of time spent in HBO_2 (LSz1 & LSz2) so that an equivalent duration of exposure to NormO_2HBHe is tested.

All experiments are conducted in Sprague-Dawley rats (male, 200-350g). The outbred/stock rat was chosen because it is the representative animal model this lab has used in its previous studies of the neurophysiology of CNS-OT [13, 15, 26-28, 39-44]. Males are used

because of the prevalence of males in U.S. Special Operations Forces and because female rats are more sensitive to HBO_2 and oxygen toxicity [27, 45], an added complexity to the study that we plan to address later in a future study.

General Approach: hyperbaric radio telemetry & EDA electrodes. All experiments will use radio telemetry (1st survival surgery, invasive) followed at least 1 week later by attachment of two EDA electrodes using either surface electrode attachment or sub-dermal attachment (2nd survival surgery, minimally invasive). 24 hours after the 2nd survival surgery, the animal will be placed in the animal chamber inside the hyperbaric chamber. Two leads will be attached to the EDA electrodes and the wires restrained by a tether harness and the animal dived to 5 ATA HBO_2 until onset of 1st and 2nd bouts of seizures. Five to 7 days later, the animal will be dived for an equivalent period using $NormO_2HBHe$ as the control for the effects of pressure per se.

Radio telemetric module implantation & EDA electrodes attachment. Each rat is transferred to the USF Hyperbaric Biomedical Research Lab and prepared for survival surgery using aseptic methods. The rat is anesthetized with isoflurane and implanted with a DSI radio telemetry module; the F-40 telemetry unit with 1 pair of biopotential leads, or alternatively the 4ET with 4 pairs of biopotential leads. The telemetry unit is implanted in the abdomen as described in the BUMED and USF Animal Use Protocols. F-40 wire leads are implanted through the skull to measure cortical EEG activity and core body temperature, whereas the 4ET module measures EEG activity, respiratory muscle electromyogram activity, electrocardiogram, and core body temperature. After recovery from surgery, rats are returned to the vivarium that same day.

At least 1 week following telemetric module implantation, each rat be anesthetized using isoflurane and the fur removed from the middle back (circular area about 2.5 cm in diameter) using a depilatory agent (Shiseido) and a pair of surface electrodes (3mm diameter) will be attached to the skin using a quick-hardening dental resin (Nissin Dental Products, Inc., MEND-REX). Alternatively, after removal of fur, a pair of Ag-Cl wires will be inserted beneath the surface of the skin (~2 mm sub-dermal) and attached using the quick-hardening dental resin. Animals will be fitted with a protective rodent jacket to prevent accidental removal of the EDA electrodes and returned to the vivarium upon recovery.

Dive protocols. The animal will be brought back to the lab and placed in the animal chamber (still wearing the protective rodent jacket). The telemetry module will be turned on via a magnet, EDA leads attached to the skin/sub-dermal electrodes. In the latter case, the point of lead attachment is secured using either liquid tape or heat shrink tubing. The animal chamber is sealed, and EDA leads are tethered and passed through the wall of the animal chamber and secured to the exterior of the animal chamber. Once sealed, the animal chamber is continuously ventilated with either air, pure oxygen (HBO_2), or 4%O_2 in helium ($NormO_2HBHe$). The animal chamber sits atop the radio telemetry receiver. The animal chamber and receiver sit inside a hyperbaric chamber that is pressurized with air or 100% N_2 in parallel with the animal chamber. Baseline telemetry recordings are made under control conditions (room air, 1 ATA) for 45-60 minutes. The two chambers are then pressurized simultaneously at a rate of 0.5 atmospheres/minute to 5 ATA. **For HBO_2 dives,** upon reaching 5 ATA, the animal chamber is switched over from air (0.2 $O_2 \times 5$

ATA = 1 ATA O_2 and 4 ATA N_2)[2] to 100% O_2 (5 ATA O_2). The [O_2] in the animal chamber is measured continuously (CWE Instruments, Gemini Respiratory Gas Analyzer) and changes quickly (< 10 sec). Time zero (t_0) for measuring LSz1 & LSz2 is marked once the fractional concentration of oxygen (F_IO_2) equals 1.0 in the animal chamber. The animal remains on HBO_2 until 2nd bout of seizures at which time is switched to air and decompressed at a rate of ~0.5 ATA/minute to avoid DCS. **For NormO$_2$HBHe dives**, upon reaching 5 ATA, the animal chamber is switched over from air (1 ATA O_2 and 4 ATA N_2) to NormO$_2$HBHe (0.2 ATA O_2 and 4.8 ATA He) and t_0 marked when F_IO_2 = 0.2 ATA O_2.[3] Total time for any given experimental session is ~1.5-3.5 hours/run. All dives are monitored in real time using a CCD video camera and recorded digitally. The animal is visualized continuously for telltale signs of CNS-OT, including tonic-clonic seizures of the upper limbs, neck and head (Table 1) coincident with increased cortical EEG activity. If the 4-ET radio transmitter is used, then additional physiological parameters monitored in real time include breathing, heart rate, and body temperature.

Measuring LSz1 & LSz2. The LSz1 and LSz2 will be determined using our modified Racine Scale of seizure behaviors [13]. The behavioral phenotype of CNS-OT in juvenile and adult Sprague-Dawley rats for seizures ranges in severity from Stage 1 through Stage 6 (Table 1). Intensity of convulsions increases with increasing seizure state number. Normal rat behaviors range from self-grooming, chewing bedding, sleeping, etc. Upon onset of cortical seizures, the rat is categorized with one of the behavioral stages listed below.

Stage	Behavior/ Phenotype
1	Freezing, staring, hard blinking and horizontal head movement
2	Hunched back, gnawing, vertical head jerks
3	Rearing with forelimbs lifted
4	Wet dog shakes
5	Rearing and forelimbs lifted with clonus
6	Generalized with forelimb and hindlimb clonus, rearing and falling

Table 1. Modified Racine Scale for behavioral seizures in Sprague-Dawley rats.

The risk for CNS-OT is measured as the LSz; specifically, first bout of seizures and second bout of seizures or LSz1 and LSz2, respectively. Two bouts of seizures are measured because the first bout is usually underwhelming in terms of intensity and duration (Stage 1), lasting only a few seconds and is then followed by normal behaviors for next several minutes to tens of minutes before the second, usually greater episode of seizures (States 2-6). If, however, the first bout of seizures is severe and long-lasting as in Stage 6 then the "dive" will be terminated at that point without waiting for the second episode of seizures.

Analysis 1. Based on our previous telemetric studies [15, 26, 28], we know that we can get useful data in 85% of the experiments. Failures (at 15% rate) may occur that require early removal of a rat from the study due to one of the following problems: death during surgery, electrode failure

[2] The brief exposure to 4 ATA N_2 is not thought to cause inert gas narcosis since much higher levels of hyperbaria are required to induce nitrogen narcosis in rats [46, 47].

[3] The brief exposure time (~4 min) to hyperoxia (>0.2 to 1.0 ATA O_2) during compression on air to maximum chamber pressure (5 ATA) is not expected to effect EDA since the dose of O_2 is sub-lethal; i.e., will not produce seizures.

post-surgery/during experimental sessions, radio-transmitter failure (transmitter battery failure), and unexpected illness/death during the study. Thus, a correction factor of 1.15 is used in animal numbers calculation to allow for 15% of animals that do not survive the experiment to completion for one of the above reasons. For these experiments, power analysis indicated a power = 0.8-0.9 for n=10 rats per group. This is based on our previous experiments using these procedures and the telemetric data generated. Using these assumptions and facts, we estimate the maximum number of animals needed for the experiments in Aim 1 as follows: 10 rats × 1.15 = 11.5 ≅ 12 rats.

GraphPad Prism version 7.04 software will be used for all statistical analyses. All values will be reported as means ± SE. As in our previous study of physio-markers during HBO_2 [15], data will not be normalized to the control values in air or 1 ATA O_2; however, because of individual variability in the latency time to seizure (LSz1 & LSz2) between animals, physiological data points will be aligned or zeroed to the onset of behavioral seizures (motor movements of the upper limbs and head; Table 1) during neurological seizure (increased EEG activity). Physiological data will be analyzed in reverse chronological order until the sample size begins decreasing as animals with the shortest-duration LSz values drop out of the pooled data. Data are analyzed by averaging the values derived from the raw data every 10 sec and a linear regression done at times 1, 2, 4, 8, and 16 mins (or similar intervals) preceding seizures 1 and 2. All statistical comparisons were considered significant at $P < 0.05$.

Anticipated Results 1. We predict that EDA will increase several minutes prior to onset of 1st seizures. During the interictal period that precedes the second seizure, we predict that EDA will remain elevated and exhibit a second surge in activity prior to the 2nd seizures. We do not expect $HBHeNormO_2$ (pressure per se) to have a significant effect on EDA. We also predict that EDA will increase in parallel with minute respiration and potentially heart rate prior to seizures.

Problems/Alternative Approaches 1. No significant problems are anticipated since we routinely conduct hyperbaric radio telemetric experiments in rats. Making EDA measurements will be new to our lab; however, the methods are already established in the literature [24] and should not be any more difficult than make a standard electrocardiogram recording.

5. General Procedures for Aim 1.

Hyperbaric Radio-Telemetry & EDA. The radio-telemetry system consists of an implantable F-40 or 4ET radio-transmitter able to amplify and broadcast signals via a receiver (DSI PhysioTel, model RPC-2) connected to an acquisition interface unit (ACQ 7700 Ponemah) via electrical penetrations in the wall of the hyperbaric chamber. The acquisition interface unit is connected to a computer for real time data collection and storage. The same acquisition unit also records chamber pressure and temperature, which are measured, respectively, by a thermocouple and pressure gauge directly connected to the acquisition system via BNC cables. Each animal will be continuously monitored via a video camera (AXIS 221 Network Camera) and the video of each experiment will be recorded as well. Raw data will be collected using DSI Ponemah software (P3 Ponemah Physiology Platform, version 4.90). ADE activity will be recorded using an ADInstruments Powerlab with GSR Amp. Statistical analysis will be performed using GraphPad PRISM®, version 7.04.

The hyperbaric system consists of two main elements: 1) a plexiglass chamber (~3 liter capacity, Diamond Box, Buxco, Electronics Inc., model PLY3114), that will house the rat during

the experiment, and 2) a hyperbaric chamber (Reimers System Inc. - 7.8 ATA MWP), that contains the plexiglass chamber and functions as the pressure vessel. Both chambers are connected to an air compressor (oil-less rotary scroll compressor – model DK6086, Powerex). The outer chamber will be pressurized using air or nitrogen (capacity ~205 liters) to minimize the risk of an electrical-induced fire.

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Future Naval Relevance

Naval unique Special Warfare/Special Operations diving maneuvers and submarine operations are limited by health risks associated with breathing various gas mixtures at normobaric and hyperbaric pressures. Included in the list of health risks is CNS-OT. Submariners must be able to survive a possible CNS-OT "hit" while breathing HBO_2 in a pressurized DISSUB emergency as they perform isobaric denitrogenation (oxygen prebreathe), to reduce the risk for DCS, in preparation of dropout decompression during a buoyant ascent. Navy divers are affected by HBO_2 because the risk of developing CNS-OT greatly limits bottom time during operations. For example, the current, highly conservative limit for breathing HBO_2 at 50 feet of sea water (fsw) is 10 minutes [9]. Research during World War II, however, showed that when divers were pushed to the onset of acute toxic symptoms of CNS-OT, including non-convulsive indications (e.g., vertigo, nausea, vomiting, dizziness) and seizures, that half of the divers could tolerate longer exposure to HBO_2 at 50 fsw. Many divers withstood at least twice as much oxygen, and some individuals tolerated exposures to HBO_2 upwards of ten times [11, 46]. The USN's goal is to expand the envelope of clandestine diving operations; that is, longer and safer dives. Currently, the only approved strategy for prevention of CNS-OT is reduction of depth (chamber pressure), and thus, P_{IO_2}, and/or the time of exposure (oxygen concentration product). One strategy currently under study, however, to extend bottom time on O_2 is to monitor the early physiological changes in a diver that precedes seizures; i.e., so-called physio-markers [11, 15]. In the present study, we plan to test whether electrodermal activity (EDA) of the sympathetic nervous system is a useful physio-marker. The proposed EDA study in rodents will be done in parallel with a study in humans at Duke University and in collaboration with University of Connecticut. This study also complements ongoing animal studies in our lab looking at other physio-markers of interest to the

USN including hyperoxic hyperventilation, heart rate and pattern, and hyperoxic hypothermia during HBO_2.

Project Schedule & Milestones

Table 2.				
Year (12 months):	Early 2019-Early 2020			
Months:	1-4		5-8	9-12
Setup EDA system & test EDA electrodes (surface vs. subdermal); JBD (b) (6)				
Implant radio transmitters & dive animals on HBO_2 & $NormO_2HBHe$; JBD, (b) (6)				
Analyze physiology data; JBD, (b) (6) & (b) (6) [redacted]				
Write manuscript; JBD with (b) (6) [redacted]				

Legend to Table 2.

JBD, Dr. Jay B. Dean (P.I. USF study); (b) (6) [redacted]

Reports & Data Deliverables

Technical Progress Reports

- written report submitted as requested by ONR Program Officier; and
- participation in the annual NAVSEA/ONR Undersea Medicine (UM) Program Review and any planned workshops.

Presentation Materials

- Viewgraphs included with each annual progress report;
- Data slide updates provided semi-annually/annually for ONR-UM Program Officer

Published manuscripts resulting from ONR-funded research.

Management Approach

1. Use of personnel. (b) (4) [redacted] We will have weekly lab meetings in which we discuss the upcoming schedule of experiments projected for the next several weeks. Care will be taken to ensure that both team members know their role in the project. Ms. Landon will be involved in radio-telemetry surgery and assist the P.I. in animal experiments and with analyzing data from the proposed experiments.

2. Government research interfaces. We plan to communicate the findings of our studies as they progress with members of the undersea medical scientific community at (b) (6) [redacted] & ONR headquarters (S. Chapman & colleagues). This is so we can receive input from the USN directly if we find it necessary to revise our protocol to better meet the needs of the U.S. Fleet or improve the study based on data and input supplied by colleagues. In addition, we

will communicate regularly with other ONR performers in academia who work in related areas of undersea medicine; for example, (b) (6) [redacted]

Facilities & Other Resources (including Government Furnished Equipment/Hardware/ Software):

(b) (4) [redacted]

[redacted] (b) (4) [redacted]

(b) (4) [redacted]

(b) (4)

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

(b) (4) [redacted]

<u>Normobaric Research Stations.</u> 1) This cell culture/brain slice electrophysiology and imaging station includes the following items*:* electrophysiology amplifier (Axoclamp 2B patch clamp amplifier); 2 Burleigh microdrives; Nikon FN1 fixed-stage microscope equipped with: DIC optics and IR video microscopy, fluorescence attachment and Roper CoolSnap camera; brain slice recording chamber; vibration isolation table; window discriminators & rate meters; 6 channel timer/ stimulator with SIU; 2 oscilloscopes; 2 channel DC amplifier/filter; Digidata 1322A/pCLAMP data acquisition system with computer; water bath; temperature controller; and fiber optic illuminator. **2)** The other cell culture/brain slice electrophysiology and imaging station includes: Nikon TE2000 inverted microscope, which is mated to a fluorescence microscopy attachment. Important pieces of equipment for fluorescence microscopy include: Roper Cool Snap Digital Camera; Xenon light source; light guide; Lambda 10-3 shutter controller and interface; Nikon Elements Software for data collection and analysis.

<u>Gas Tank Farm</u>: A 528 ft^2 cement patio, enclosed by chain-link & barbed-wire fencing and overhanging roof, houses the following items used to compress/decompress the pressure chambers: oil-less scroll air compressor (model SES; MWP 115 PSIG) and a vertical air handling tank (400 gallons, 93" tall x 36" dia.; MWP 137 PSIG); 1-HP oil-less rotary vane vacuum pump (30 gallons tank) is plumbed to two chambers (b) (6) [redacted]) for producing altitudes over 20,000 feet; and space for up to 60 K-type gas bottles; and gas-mixing station for blending customized gas mixtures. A dual cylinder horizontal roller is used to mix gases cylinders that may have separated out. Compression and vacuum lines penetrate the back wall and terminate on the interior wall of MDL1026 with a manifold control panel so that helium, nitrogen or air can be directed to anyone one of up to six different work stations. In addition, various cylinders of O_2, CO_2 & N_2/He are plumbed from the tank farm to each work station for pressurizing sample cylinders used to produce HBO_2 media.

Histology: A full service research histology laboratory is available for histological and cytological preparations, tissue array construction, immunostaining and in situ hybridization in the Dept. of Molecular Pharmacology and Physiology (M. Barter). A Dako automated immunostainer, a Chemicon ATA 100 tissue arrayer and 2 cryostats as well as the equipment items necessary for paraffin processing, sectioning and staining are available.

Biostatistics Shared Resource: The Biostatistics Shared Resource staffs two doctoral-level biostatisticians available for consultation on all statistical aspects of research studies including: study design, database design, sample-size analysis, data analysis and interpretation, statistical/software instruction and manuscript writing. Available software tools include: SAS, SPSS, S-Plus, R, and nQuery.

Computer: Each research station maintains 1-2 computers for online data collection and offline data analysis. In addition, each office (3 total: P.I., lab manager & trainees) maintains a computer for word processing and each member of the lab owns a lap top computer.

Other: BioTek ELX800 Spectrophotometer (BioTek Instrument) for biochemical assays; Kerr Tissue Recording System for cell or tissue electrophysiology recordings if needed. General lab equipment includes: deionized water still; water baths, 2 Mettler analytical balances; pH meters; sonicators and refrigerators and freezers, including a -80°F freezer. Also available is additional equipment used for brain slicing and fabrication of microelectrodes and micropipettes: 3 micropipette pullers; Nikon Labophot & Optiphot microscopes; Nikon patch pipette polisher; Zeiss microscope with phase contrast optics; 3 Pelco Vibratomes for making tissue slices.

Animal: The USF Division of Comparative Medicine (DCM) serves as the advocate for animals involved in research at USF, and provides a fully accredited, centralized service of pathogen-free animal procurement, husbandry, health surveillance, and quality control. DCM is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC #000434). Its program and facilities for animal care and use are managed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, the PHS Policy on Humane Care and Use of Laboratory Animals (PHS assurance #A4100-01), and the Animal Welfare Regulations. DCM has an assurance of compliance on file with OLAW and NIH, and is a registered research facility with the United States Department of Agriculture (USDA registration #58-R-0015). Animals are purchased as needed through Huntingdon Laboratories (formerly Harlan Laboratories).

Cell Culture: Available if needed. P.I. has just received cell culture incubator and hood from the former NEDU electrophysiology laboratory. In addition, we have access to departmental shared cell culture facility 327 ft^2 and two specialized cell culture incubators (Galaxy 48R, New Brunswick Instruments) that are capable of maintaining cell cultures under a wide range of oxygen concentrations (1% to 95%). is arranging for HBRL to acquire a new cell culture facility in addition to the shared facility currently available.

Office: The Department of Molecular Pharmacology and Physiology provides staff support for this project, including the following: (b) (4), (b) (6) [redacted]

[redacted] (b) (4) [redacted]

Other Aspects of the Research Environment/Facilities: The College of Engineering supports an instrument shop, which the PI has access to at the cost of $50/hr labor plus materials. The PI also has access (fee basis) to an assortment of machinists/model makers and electricians in the Tampa Bay area who do work for hire with USF faculty. (b) (4) [redacted] A full-time PhD degree program is available with various research tracks including Neuroscience and Physiology.

Current and Pending Project and Proposal Submissions

Jay B. Dean, Ph.D.

Project 1, JBD (PI)—the proposed study 3/2019 - 2/2020

1) Title of Proposal and Summary: Animal studies, Aim 1 (USF Tampa): Feasibility of measuring electrodermal activity for detecting seizures elicited by central nervous system oxygen toxicity. The goal of this project is to determine if electrodermal activity (skin electrical resistance) changes significantly in a small animal model before onset of seizures while breathing hyperbaric oxygen (HBO_2). If so, then increased electrodermal activity is a possible physiological marker of an impending seizure otherwise known as CNS oxygen toxicity, CNS-OT.

2) Source and amount of funding:

Total annual direct costs: $16,707

Grant No.: this proposal

3) Percentage effort devoted to project—2%

4) Identity of prime Offeror—ONR Undersea Medicine

5) Technical contact—P.I.: Jay B. Dean, Ph.D.; 813-974-1547/813-905-9965; jdean@health.usf.edu

6) Administrative/business contact—Sandra Chapman, Ph.D.; Undersea Medicine Program Officer; Warfighter Performance Department (Code 34); Office of Naval Research; 875 N Randolph St.; Arlington VA 22203; (703) 588-2429; Sandra.chapman@navy.mil

7) Period of performance—2/2019 through 1/2020

8) The proposed project and all other projects or activities requiring a portion of time of the Principal Investigator and other senior personnel must be included, even if they receive no salary support from the project(s)

P.I., J.B. Dean—2%

(b) (6) [redacted] —9%

9) The total award amount for the entire award period covered (including indirect costs, IDC) must be shown as well as the number of person-months or labor hours per year to be devoted to the project, regardless of source of support

Total direct costs + Total IDC = $16,707 + $8,270 = $24,977

10) State how projects are related to the proposed effort and indicate degree of overlap.—this is the proposed study

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Project 2, JBD (PI) 6/1/2016—5/31/2019 (NCE August 2019)

1) Title of Proposal and Summary: Cellular mechanisms of CNS oxygen toxicity during CO_2 retention and ketone metabolic therapy. The goal of this research project is to further understand how conditions that accelerate and delay onset of CNS oxygen toxicity (CNS-OT) seizures affect each of the following phenomena that are implicated in CNS-OT in rodents: i) increased production of reactive O_2 and N_2 species \ in the brain stem; ii) electrical signaling by medullary neurons; and iii) respiration during the period

preceding Sz; i.e., hyperoxic hyperventilation. iv) An additional goal is to determine the effects of chronic CO_2 retention on cardiorespiratory and gastric physiology.

2) Source and amount of funding:
Total annual direct costs: $219,933
Grant No.: ONR N00014-16-1-2537

3) Percentage effort devoted to project: 30%

4) Identity of prime offeror: ONR Undersea Medicine
5) Technical contact—P.I.: Jay B. Dean, Ph.D.; 813-974-1547/813-905-9965; jbdean813@gmail.com
6) Administrative/business contact—Sandra Chapman, Ph.D.; Undersea Medicine Program Officer; Warfighter Performance Department (Code 34); Office of Naval Research; 875 N Randolph St.; Arlington VA 22203; (703) 588-2429; Sandra.chapman@navy.mil
7) Period of performance—6/1/2016 through 5/31/2019 plus requesting NCE through August 2019
8) The proposed project and all other projects or activities requiring a portion of time of the Principal Investigator and other senior personnel must be included, even if they receive no salary support from the project(s)
P.I., J.B. Dean—30%
(b) (6) [redacted] —60%
9) The total award amount for the entire award period covered (including indirect costs, IDC) must be shown as well as the number of person-months or labor hours per year to be devoted to the project, regardless of source of support
Total direct costs + Total IDC = $977,569
10) State how projects are related to the proposed effort and indicate degree of overlap.—there is no scientific overlap

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Project 3, JBD (co-I) 2018-2021
1) Title of Proposal and Summary: Optimizing ketone metabolic therapy and identifying biomarkers for mitigation and prediction of CNS oxygen toxicity: animal studies. The major goal of this project is to define the efficacy and anticonvulsant mechanism of ketones using in vivo and in vitro models
2) Source and amount of funding:
Total annual direct costs: $228,559
Grant No.: ONR N00014-18-1-2701
3) Percentage effort devoted to project: 10%
4) Identity of prime offeror: ONR Undersea Medicine
5) Technical contact—(b) (6) [redacted]
6) Administrative/business contact—Sandra Chapman, Ph.D.; Undersea Medicine Program Officer; Warfighter Performance Department (Code 34); Office of Naval Research; 875 N Randolph St.; Arlington VA 22203; (703) 588-2429; Sandra.chapman@navy.mil
7) Period of performance—8/1/2018 through 7/31/2021
8) The proposed project and all other projects or activities requiring a portion of time of the Principal Investigator and other senior personnel must be included, even if they receive no salary support from the project(s)
P.I., J.B. Dean—10%
(b) (6) [redacted] —20%
9) The total award amount for the entire award period covered (including indirect costs, IDC) must be shown as well as the number of person-months or labor hours per year to be devoted to the project, regardless of source of support
Total direct costs + Total IDC = $1,021,278

10) State how projects are related to the proposed effort and indicate degree of overlap.—there is no scientific overlap

(b) (6) [redacted]
No extramural funding to report

Qualifications: See attachments

Jay Dean, PI, 2% effort: Dr. Dean is the Director of the USF Hyperbaric Biomedical Research Laboratory. He takes overall responsibility for the performance of the study. He will conduct, in collaboration with his laboratory personnel, all the proposed experiments, assist in the analysis of the data, direct writing of papers, and deliver data presentations at national/international meetings and symposia. Dr. Dean's technical expertise is studying the electrophysiology and oxidative neurobiology of mammalian neurons in reduced animal and whole animal models (rat). Dr. Dean has employed a variety of technical approaches including hyperbaric electrophysiology (in vitro), hyperbaric microscopy (in vitro), hyperbaric radio-telemetry and plethysmography (in vivo) and other instrumented approaches in rats to study the effects of normobaric gases and hyperbaric gases at cellular and organismal levels.

(b) (6) [redacted] will provide technical support to the laboratory of Dr. Dean, including radio-telemetry surgery, animal maintenance, solution preparation, ordering of supplies, budget maintenance, data analysis and figure preparation, including computer graphics work. She is greatly experienced with all aspects of laboratory management.

Biographical Sketches follow on next pages…

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Jay B. Dean, PhD	POSITION TITLE Professor of Molecular Pharmacology & Physiology
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Central Michigan University, Mt. Pleasant, MI	B.S.	8/79	Biology, Chemistry
Michigan Technological Univ., Houghton, MI	M.S.	5/81	Biol. Sci. (Respiratory Control)
The Ohio State University, Columbus, OH	Ph.D.	12/86	Physiology (Control of body temperature)
Univ. of North Carolina, Chapel Hill, NC	Post-Doc.	12/86-11/91	Neurophysiology (Resp.Control)

A. Personal Statement

My lab studies the effects of O_2, CO_2 and barometric pressure on the function of the mammalian CNS, with an emphasis on the cardio-respiratory and gastro-esophageal control systems. Ongoing research focuses on understanding how hyperoxia and hypercapnia interact to generate reactive O_2 and N_2 species that, in turn, activate neuronal activity in brain stem neurons. This research has relevance to understanding normal and abnormal function in cardio-respiratory control during periods of disordered breathing, environmental stresses, and in understanding neurological problems arising from the use of hyperoxia in clinical medicine and combat operations. We are particularly interested in the following: 1) neurophysiology of CNS oxygen toxicity (seizures) and its mitigation by ketone metabolic therapy; 2) understanding how CO_2 and H-ions are "sensed" by chemoreceptor neurons in the caudal medulla oblongata; and 3) understanding how gastro-esophageal CO_2 ventilation supplements pulmonary ventilation during acute and chronic respiratory acidosis, digestion and other conditions that stimulate gastric acid secretion.

B. Positions and Honors

Positions and Employment

1986-88	NINCDS Postdoctoral Fellow, Dept. of Physiology, Curriculum in Neurobiology, UNC-CH (D.E. Millhorn)
1988-90	NRSA Postdoctoral Fellow, Dept. of Physiology, UNC-CH (D.E. Millhorn)
1990-91	Res. Assist. Prof./Parker B. Francis Fellow in Pulmonary Res., Dept. of Physiology, UNC-CH (D.E. Millhorn)
12/91-97	Assistant Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
1997-02	Associate Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
7/02-6/03	Professor, Dept. of Physiology & Biophysics, Wright State University, Dayton, OH
7/03-10/04	Acting-Chairman, Dept. of Anatomy & Physiology, Wright State Univ., Dayton, OH
11/04-3/05	Vice Chair of Administration, Dept. of Anatomy & Physiology, Wright State Univ., Dayton, OH

3/05-6/06 Vice Chair of Administration, Dept. of Neuroscience, Cell Biology and Physiology, WSU, Dayton, OH
7/1/06-pres. Professor, Dept. of Molecular Pharmacology & Physiology, Coll. of Med., Univ. of South Florida, Tampa, FL
7/1/10-10/30/11 Acting Chair, Molecular Pharmacology & Physiology, Coll. of Med., Univ. of South Florida, Tampa, FL

Other Experience and Professional Memberships

1992-94 NSF, Special Emphasis Panel for Research Planning Grants and Career Advancement Awards for Women
1998 NIH, Special Emphasis Panel
2000 NIH, Respiratory and Applied Physiology Study Section, Temporary Member
2002 NIH, Respiratory Physiology Study Section, Ad-hoc Member
3/2003 NIH grant review panel for SCOR grants, Sleep Apnea
10/03-6/07 Regular member, NIH Respiratory Integrative Biology and Translational Research Study Section (RIBT)
1999-00 Co-organizer & Lecturer, International Symp., Central Chemoreception 2000, Ruhr Univ., Bochum, Germany
2001-02 Co-chair & Lec., Featured Topic, EB2002, Role of gap junctions in CO_2-chemoreception & respiratory control
2000-01 Guest Co-Ed., Special Issue, Respir. Physiol., Central Chemoreception 2000 (vol. 129:1-278, 2001)
2003-04 Chair & Lecturer, Featured Topic, EB 2004, Oxygen Sensing by Neural Tissues
4/10-6/10 Co-Ed., Highlighted Topic, J. Appl. Physiol., Central CO_2 Chemoreception & Cardio-respiratory Control
1999-pres. Editorial Board, Journal of Applied Physiology
2008-2010 Associate Editor, Journal of Applied Physiology (Hyperbaric Physiology, Respiratory Control)
2002- 2013 Editorial Board, Undersea and Hyperbaric Medicine
1987- 2011 Member, Society for Neuroscience
1997- pres. Member, American Physiological Society
1997- 2009 Member, Undersea and Hyperbaric Medical Society
2000- 2013 Member, Society for Free Radical Biology and Medicine
2004- pres. Member, Aerospace Medical Association

Honors

7/03-6/06 Brage Golding Distinguished Professor of Research, Wright State Univ., Dayton, OH

Selected peer-reviewed publications pertinent to this application (from a total of >60)

Ciarlone, G.E., and Dean, J.B. (2019) Acute hyperoxia increases CO_2-chemosensitivity in rat caudal solitary complex neurons. American Journal of Physiology, Cell Physiology (in revision; manuscript C-00368-2018R1)

Ciarlone, G.E., Hinojo, C.M., Stavitzski, N.M., and Dean, J.B. (2019) CNS function and dysfunction during exposure to hyperbaric oxygen in operational and clinical settings. Invited review for Redox Biology, Special Issue entitled, “Redox Biology in Physiology and Disease” (accepted pending minor revisions; manuscript no. REDOX_2019_115)

Ari, C., Koutnik, A. P., DeBlasi, J., Landon, C. S., Rogers, C. Q., Vallas, J., Bharwani, S., Puchowicz, M., Bederman, I., Diamond, D.M., Kindy, M.S., Dean, J.B., and D'Agostino, D. P. e. (2019). Delaying latency to hyperbaric oxygen-induced CNS oxygen toxicity seizures by combinations of exogenous ketone supplements. Physiological Reports. doi: 10.14814/phy2.13961

Morris K.F., Nuding S.C., Segers L.S., Iceman K.E., O’Connor R., Dean J.B., Ott M.M., Alencar P.A., Shuman D., Horton K.K., Taylor-Clark T.E., Bolser D.C., and Lindsey B.G. (2017) Carotid

chemoreceptors tune breathing via multipath routing: Reticular chain and loop operations supported by parallel spike train correlations. J Neurophysiol 119(2):700-722. doi: 10.1152/jn.00630.2017

Ciarlone G. E. and Dean J. B. (2016a) Normobaric hyperoxia stimulates superoxide and nitric oxide production in the caudal solitary complex of rat brain slices. Am J Physiol Cell Physiol 311: C1014–C1026,

Ciarlone G. E. and Dean J. B. (2016b) Acute hypercapnic hyperoxia stimulates reactive species production in the caudal solitary complex of rat brain slices but does not induce oxidative stress. Am J Physiol Cell Physiol 311: C1027–C1039, 2016.

Gianni D'Angelo, Raffaele Pilla, Jay B. Dean, Salvatore Rampone (2016) Toward a soft computing-based correlation between oxygen toxicity seizures and hyperoxic hyperpnea. Soft Computing (METHODOLOGIES AND APPLICATION), DOI 10.1007/s00500-017-2512-z

Matott, M.P., Ciarlone, G.E., Putnam, R.W., and Dean, J.B. (2014) Normobaric hyperoxia (95% O_2) stimulates CO_2-sensitive and CO_2-insensitive neurons in the caudal solitary complex of rat medullary tissue slices maintained in 40% O_2. Neuroscience 270: 98-122

Held, H.E., Pilla, R., Ciarlone, G.E., Landon, C.S., Dean, J.B. (April 2014) Female rats are more susceptible to central nervous system oxygen toxicity than male rats. Physiological Reports 2 (e00282) DOI: 10.14814/phy2.282; http://physreports.physiology.org/content/2/4/e00282.short

Pilla, R., Held, H.E., Landon, C.S., and Dean, J.B. (2013) High doses of pseudoephedrine hydrochloride accelerate onset of CNS oxygen toxicity seizures in unanesthetized rats. Neuroscience 246: 391-396.

D'Agostino, D.P., Pilla, R., Held, H.E., Landon, C.S., Puchowicz, M., Brunengraber, H., Ari, C., Arnold, P. and Dean, J.B. (2013) Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. Am. J. Physiol. Reg., Integr. and Comp. Physiol. 304(10): R829-R836

Pilla, R., Landon, C.S., and Dean, J.B. (2013) A potential early physiological marker for CNS oxygen toxicity: hyperoxic hyperpnea precedes seizure in unanesthetized rats breathing hyperbaric oxygen. J. Appl. Physiol. 114: 1009-1020.

D'Agostino, D. P., McNally, H.A., and Dean, J.B. (2012) Development and testing of hyperbaric atomic force microscopy (AFM) and fluorescence microscopy for biological applications, J. Microscopy 246(2): 129-142

Dean J.B. (2011) Theory of gastric CO_2 ventilation and its control during respiratory acidosis: Implications for central chemosensitivity, pH regulation, and diseases causing chronic CO_2 retention, Respir. Physiol. Neurobiol. 175(2): 189-209; with Commentary by Forster H.V. (2011) Gastric acid secretion contributes to elimination of CO_2 during hypercapnia, Respir. Physiol. Neurobiol. 175: 189-209

Dean, J.B. Putnam, R.W. (2010) The caudal solitary complex is a site of central CO_2 chemoreception and integration of multiple systems that regulate expired CO_2, Resp. Physiol. & Neurobiol. 173(3): 274-287.

Garcia III, A.J., Putnam, R.W. and Dean, J.B. (2010) Paper 1 of 2: Hyperbaric hyperoxia and normobaric reoxygenation increase excitability and activate oxygen-induced potentiation (OxIP) in CA1 hippocampal neurons. J. Appl. Physiol. 109: 804-819

Garcia III, A.J., Putnam, R.W. and Dean, J.B. (2010) Paper 2 of 2: Hyperoxic stimulation of synchronous orthodromic activity and induction of neural plasticity does not require changes in excitatory synaptic transmission. J. Appl. Physiol. 109: 820-829

Dean JB. (2010) Hypercapnia causes cellular oxidation and nitrosation in addition to acidosis: implications for CO_2 chemoreceptor function and dysfunction, J. Appl. Physiol. 108(6):1786-1795

Nichols, N.L., Wilkinson, K.A., Powell, F.A., Dean, J.B. and Putnam, R.W. (2009) Chronic hypoxia suppresses the CO_2 response of solitary complex (SC) neurons from rats. Respir. Physiol. & Neurobiol., 168:272-280

D'Agostino, D.P., Olson, J.E., and Dean, J.B. (2009) Acute hyperoxia increases lipid peroxidation and induces plasma membrane b lebbing in human U87 glioblastoma cells. Neuroscience 159: 1011-1022

Dean, J.B. and D'Agostino, D. P. (2008) Chapter 10. Pressure effects on human physiology. In: Physiology and Medicine of Hyperbaric Oxygen Therapy (edited by Tom S. Neuman and Stephen R. Thom), Elsevier Inc., Philadelphia, pp. 189-202

D'Agostino, D.P., Colomb, D.G., and Dean, J.B. (2009) Effects of hyperbaric gases on membrane nanostructure and function in neurons. J. Appl. Physiol. 106: 996-1003

D'Agostino, D.P., Putnam, R.W., and Dean, J.B. (2007) Superoxide ($\cdot O_2^-$) production in CA1 neurons of rat hippocampal slices exposed to graded levels of oxygen. *J. Neurophysiol.* 98: 1030 – 1041.

Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Pressure ($\leq$4 ATA) increases membrane conductance and firing rate in the rat solitary complex, *Journal of Applied Physiology*, 95(3): 922-930

Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Hyperbaric oxygen and chemical oxidants stimulate CO_2/H^+-sensitive neurons in rat brain stem slices, *Journal of Applied Physiology*, 95 (3): 910-921

Dean, J.B., Mulkey, D.K., Garcia III, A.J., Putnam, R.W., and Henderson III, R.A. (2003) Neuronal sensitivity to hyperoxia, hypercapnia, and inert gases at hyperbaric pressures, *Journal of Applied Physiology* 95(3): 883-909

Dean, J.B., Mulkey, D.K., Henderson III, R.A., Potter, S.J., and Putnam, R.W. (2004) Hyperoxia, Reactive O_2 Species, and Hyperventilation: O_2-Sensitivity of Brain Stem Neurons, *Journal of Applied Physiology*, 96: 784-791.

Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2004) Oxidative stress decreases intracellular pH, Na^+/H^+ exchange and increases excitability of solitary complex neurons from rat brain slices, *American Journal of Physiology, Cell Physiology*, 286: C940-C951.

Mulkey, D.K., Henderson III, R.A., Ritucci, N.A., Putnam, R.W., and Dean, J.B. (2004) Chemical oxidants acidify solitary complex (SC) neurons in rat. In: *Undersea and Hyperbaric Medicine*, 31(1): 107-111

Ritucci, N.A., Dean, J.B., and Putnam, R.W. (2005) Somatic versus dendritic responses to hypercapnia in chemosensitive locus coeruleus neurons from neonatal rats. *Am. J. Physiol. Cell Physiol.*, 289: C1094-C1104

Dean, J.B. and Mulkey, D.K. (2000) Continuous intracellular recording from mammalian neurons exposed to hyperbaric helium, oxygen or air, *Journal of Applied Physiology* 89:807-822.

Mulkey, D.K., Henderson III, R.A., Olson, J.E., Putnam, R.W., and Dean, J.B. (2001) Oxygen measurements in brainstem slices exposed to normobaric and hyperbaric oxygen, *Journal of Applied Physiology*, 90: 1887-1899

D. Research Support

Ongoing Research Support

ONR N00014-16-1-2537 Dean (PI) 6/1/2016 – 5/31/2019
(no cost extension to be requested: -8/31/2019)

Office of Naval Research, Undersea Medicine Program

Cellular mechanisms of CNS oxygen toxicity during CO_2 retention and ketone metabolic therapy

The goal of this research project is to further understand how conditions that accelerate and delay onset of CNS oxygen toxicity (CNS-OT) seizures (Sz) affect each of the following phenomena that are implicated in CNS-OT in rodents: i) increased production of reactive O_2 and N_2 species \ in the brain stem; ii) electrical signaling by medullary neurons; and iii) respiration during the period preceding Sz; i.e., hyperoxic hyperventilation. iv) An additional goal is to determine the effects of chronic CO_2 retention on cardiorespiratory and gastric physiology.

(b) (6), (b) (4) [redacted]

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
(b) (6) [redacted]	Laboratory Manager/ Senior Biological Scientist
eRA COMMONS USER NAME N/A	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
(b) (6) [redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

A. Positions

(b) (6) [redacted]

Honors

(b) (6) [redacted]

B. Publications

(b) (6) [redacted]

(b) (6), (b) (4) [redacted]

VOLUME 2: Cost Proposal

Part 1: Itemized Budget

Next Page

Proposal Budget Worksheet

Principal Investigator:	Jay Dean
Sponsoring Agency:	University of Conneticut
Project Title:	Electrodermal activity during hyberbaric oxygen exposure rat study
Start Date:	2/1/19
End Date:	1/31/20
Grant Years:	1

NIH Salary Cap	Fringe Options	Fringe Rate	Health Insurance	Fiscal Year 2018	Life Insurance
(b) (4)	Family	(b) (4) %	(b) (4)	Annual	(b) (4)
COL Escalation	Spouse	(b) (4) %	$(b) (4)	Annual	Type Appt
0%	Individual	(b) (4) %	(b) (4)	Annual	12
Tuition / Hour	No Health	(b) (4) %	(b) (4)	Annual	9
(b) (4)	Temporary	(b) (4) %	(b) (4)	Annual	3
Escalation	Post Doc	(b) (4) %	(b) (4)	Annual	9&3
(b) %	Student	(b) (4) %	(b) (4)	Annual	6

	ROLE ON PROJECT	TYPE APPT (3,9,12)	EFFORT ON PROJ	PERSON MONTHS Cal	Acad	Smmr	BASE SALARY	TYPE OF FRINGE	Year 1	Year 2	Year 3	Year 4	Year 5	ALL YRS
A. Key Personnel														
Jay Dean	PI	12	2.00%	0.24	0	0	$(b) (4)	Family	(b) (4)	$0	$0	$0	$0	(b) (4)
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
								Key Personnel Total	$(b) (4)	$0	$0	$0	$0	$(b) (4)
B. Other Personnel														
Carol Landon	Lab Manager	12	9.00%	1.08	0	0	$(b) (4)	Spouse	$(b) (4)	$0	$0	$0	$0	(b) (4)
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
			0.00%	0	0	0			$0	$0	$0	$0	$0	$0
								Other Personnel Total	(b) (4)	$0	$0	$0	$0	$(b) (4)
Total Personnel									$11,273	$0	$0	$0	$0	$11,273
							# of Students	0						
C. Equipment			(no F&A if Federal)						$0	$0	$0	$0	$0	$0
D. Travel														
	1. Domestic								$0	$0	$0	$0	$0	$0
	2. International								$0	$0	$0	$0	$0	$0
E. Participant/Trainee Costs									$0	$0	$0	$0	$0	$0
F. Other Direct Costs														
	1. Materials and Supplies								$820	$820	$0	$0	$0	$1,640
	2. Publication Costs								$0	$0	$0	$0	$0	$0
	3. Consultant Costs								$0	$0	$0	$0	$0	$0
	4. ADP/Computer Services								$0	$0	$0	$0	$0	$0
	6. Equipment/Facility Rental								$0	$0	$0	$0	$0	$0
	7. Alterations/Renovations								$0	$0	$0	$0	$0	$0
	8. Other		Telemetry (refurbish and batteries)						$2,444	$2,444	$0	$0	$0	$4,888
	9. Other		Animal Costs						$1,000	$1,000	$0	$0	$0	$2,000
	10. Other		GSR Amp for AD Instruments						$1,170	$1,170	$0	$0	$0	$2,340
	5. Subaward/Consortium													
	Subaward 1 <25K						0		$0	$0	$0	$0	$0	$0
	Subaward 1 >25K		(no F&A if Federal)						$0	$0	$0	$0	$0	$0
	Subaward 2 <25K						0		$0	$0	$0	$0	$0	$0
	Subaward 2 >25K		(no F&A if Federal)						$0	$0	$0	$0	$0	$0
	Subaward 2 <25K						0		$0	$0	$0	$0	$0	$0
	Subaward 2 >25K		(no F&A if Federal)						$0	$0	$0	$0	$0	$0
			Subaward Total						$0	$0	$0	$0	$0	$0
Total Other Direct Costs									$5,434	$5,434	$0	$0	$0	$10,868
Total Direct Costs									$16,707	$5,434	$0	$0	$0	$22,141

*No tuition required for MCDM students

*Please note that for federal grants consortium F&A is not included in the direct cost limitations

	Year 1	Year 2	Year 3	Year 4	Year 5	ALL YRS
Total Direct Costs	$16,707	$5,434	$0	$0	$0	$22,141
DIRECT w/o subs F&A	$16,707	$5,434	$0	$0	$0	$22,141
BASE ● MTDC ○ TDC	$16,707	$5,434	$0	$0	$0	$22,141
F&A COSTS	$8,270	$2,690	$0	$0	$0	$10,960
TOTAL COSTS	$24,977	$8,124	$0	$0	$0	$33,101
F&A RATE	(b) (4)					
Target (direct costs)	$25,000	$0	$0	$0	$0	$25,000

Direct Labor:
Budget Justification.

1) Personnel salaries, wages and fringe benefits (% effort).

Please see the attached computer-generated budget pages at the end of this section. A breakdown of the salaries per investigator per year is also provided.

Budget justification:

Key personnel—($11,273/yr 1) Jay B. Dean (PhD; 2% effort) is a Co-I on Dr. Chon's project and the lead investigator of the subcontract at USF Tampa for Aim 1 (animal experiments). Dr. Dean is director of the USF Hyperbaric Biomedical Research Laboratory and an expert in hyperbaric physiology and neurophysiology. Dr. Dean will take responsibility for all the proposed animal experiments in Aim 1, which measures EDA before and during onset of CNS-OT seizures in rats. (b) (6) [redacted] (BS; 9% effort) is the lab manager for the USF Hyperbaric Biomedical Research Laboratory (b) (6) [redacted] is involved in all animal experiments including surgery to implant the radio telemetry module and in assisting the Co-I in conducting and analyzing the experiments.

Materials and supplies—($820/yr 1) Funds are requested to purchase surgical supplies, anesthetics and analgesics, and gases that are used for implanting radio-telemetry modules and EDA leads under sterile conditions. Gases are also used for administering HBO to induce CNS-OT.

Other: telemetry ($2,444/yr 1), animal costs ($1,000/yr 1), and GSR Amp for ADInstruments data acquisition system ($1,170/yr 1)—Funds are requested to have Data Sciences International (DSI) refurbish four F-40 radio-telemetry modules that we currently have in the lab. Funds are also requested for 12 animals, which includes purchase price plus shipping and per diem costs. Finally, a GSR amplifier that interfaces with a preexisting ADInstruments data acquisition system is requested to measure EDA in rats.

5) Indirect Costs

University of South Florida has a modified indirect rate of ((b) (4) [redacted]% × total direct costs). Please see attached budget page.

6) Total cost

$24,977

Chapter One

[1] UHMS, Indications for Hyperbaric Oxygen Therapy, in: L.K. Weaver (Ed.) Undersea and Hyperbaric Medical Society Hyperbaric Oxygen Therapy Indications, Undersea and Hyperbaric Medical Society, Durham, North Carolina, 2018.
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[13] C.M. Hinojo, N.M. Stavitzski, C.S. Landon, J.B. Dean, Reevaluation of CNS oxygen toxicity seizures in male Sprague-Dawley Rats, FASEB Journal, 31 Supplement (2017) 711.716.
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[15] R. Pilla, C.S. Landon, J.B. Dean, A potential early physiological marker for CNS oxygen toxicity: hyperoxic hyperpnea precedes seizure in unanesthetized rats breathing hyperbaric oxygen, Journal of applied physiology (Bethesda, Md. : 1985), 114 (2013) 1009-1020.
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[17] B.A. Reyes, H.F. Posada-Quintero, J.R. Bales, A.L. Clement, G.D. Pins, A. Swiston, J. Riistama, J.P. Florian, B. Shykoff, M. Qin, K.H. Chon, Novel electrodes for underwater ECG monitoring, IEEE Transactions on Biomedical Engineering, 61 (2014) 1863 - 1876.
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BUMED Animal Use Appendix for Research Involving Animals
ABBREVIATED VERSION

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a. A copy of their **IACUC-approved institutional protocol(s)** (BUMED will ONLY review **approved** protocols) and documentation of IACUC approval

b. A copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by BUMED **PRIOR** to implementation)

c. A completed Appendix for each IACUC-approved protocol.

This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs.

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DoD regulations. The DoD policies and requirements for the use of animals in DoD-supported research, development, testing and evaluation are described in DoD Instruction 3216.01, dated September 13, 2010 and SECNAVINST 3900.38C, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to BUMED Veterinary Affairs:

Phone: (b) (6)(b) (6)(b) (6)(b) (6) [redacted]
Email: DON – VRPP, (b) (6) [redacted]
Mail: Department of the Navy Bureau of Medicine and Surgery
ATTN: Director for Veterinary Affairs
Bldg 1564 Room 122
1564 Freedman Drive
Fort Detrick, MD 21702

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Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text. Submit electronic copies of the appendix only; please do not submit printed copies to BUMED. Any section of the Appendix that is not applicable to your proposal, e.g., no surgery or no prolonged restraint, should be marked "**No**" or "**N/A**". There are no space limitations for the responses.

It is essential that only animal studies or procedures documented in an IACUC–approved protocol or amendment be performed at your facility. BUMED will collect animal usage information for end-of-year DoD animal use reports separate from the grant annual progress reporting requirement throughout the life of your award. For this reason, Principal Investigators or other delegated research personnel should keep accurate records and be able to provide an audit trail of all animal use that correlates to their approved protocol.
~~~~~~

[X] Yes [ ] No All animal studies described in the attached IACUC-approved protocol are funded by this DOD award/contract. If no, STOP ! You must use the **full version** of the Animal Use Appendix.

1. Administrative Data: (Provide Attending Vet, IACUC, and Research Office info for the work site.)

DoD/Contract PI Name: Jay B. Dean, PhD

Grant/Contract PI Email: [redacted] **Phone:** [redacted]

Protocol PI Name: Jay B. Dean, PhD

Protocol PI Email: [redacted] **Phone:** [redacted]

Animal Research Site (RS): University of South Florida, Tampa, FL

RS Attending Veterinarian: [redacted]

Attending Vet Email: [redacted] **Phone:** [redacted]

RS IACUC Point of Contact (POC): [redacted]

IACUC POC Email: [redacted] **Phone:** [redacted]

RS Grants Office Point of Contact (POC): [redacted]

Grants Office POC Email: [redacted] **Phone:** [redacted]

Animal Protocol Title: Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels

2. Total Number of Animals Used (by Species) and USDA Pain/Distress Category:

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Sprague-Dawley rat	C	25
Sprague-Dawley rat	C	122
Sprague-Dawley rat	E	305
Mouse: Aquaporin-4 KO	E	20
Mouse: C57BL/6 WT	E	50

USDA Pain/Distress Category Definitions:

- ***Column B:*** *Animals being bred, and animals being held for use in research, testing, teaching, experiments or surgery but not yet used for those purposes.*
- ***Column C:*** *List the number of animals that will experience no more than slight or momentary pain or distress as a result of experimental manipulations or procedures on this protocol.*

- ***Column D:*** *List the number of animals that will potentially experience more than momentary or slight pain or distress that* ***WILL*** *be alleviated through the use of anesthetics and/or analgesics.*

- ***Column E:*** *List the number of animals that will experience more than momentary or slight pain or distress that* ***WILL NOT*** *be alleviated or relieved with anesthetics or analgesics.* **If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:**. .

  - USDA Pain Category E (= pain category C in USF IACUC animal use protocol): Animals listed in USDA Pain Category E will be exposed to hyperbaric oxygen until onset of two bouts of seizures, which may cause discomfort. The potential for discomfort is why these animals are placed in USDA pain category E. The animals are unanesthetized in these experiments so that their visible behaviors can be used as one of the criteria to score onset of a seizure; we use a six-point scale of behavior to evaluate the type of seizures. Sensitivity to hyperbaric oxygen is blunted in an anesthetized animal and thus the latency time to seizures is severely lengthened plus a higher oxygen pressure is required. After the second bout of seizures occurs the breathing atmosphere is changed from 100% O_2 to air and the animal chamber is decompressed at a rate of ~1 atmosphere/minute. The 1st bout of seizures is short lasting (seconds) and during the interictal period before the 2nd bout of seizures the animal, remarkably, appears fine for all intents and purposes based on its behavior. After recovery at room pressure, the animal appears normal based on its behavior.

3. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals. Note the DoD-specific database requirements in subparagraph a.

a. Literature Source(s) Searched:

http://www.ntis.gov/products/fedrip.aspxhttp://projectreporter.nih.gov/reporter.cfm

☐ Federal Research in Progress (FEDRIP) http://www.ntis.gov/products/fedrip.aspx

or

☒ Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER) http://projectreporter.nih.gov/reporter.cfm

b. Date of Search: 16-17 December 2019

c. Years Covered By Search: 1994-2019

d. Key Words and/or Search Strategy:

Search 1: ((mitochondria targeted antioxidant therapy) or MitoQ or Mitoquinone), hyperbaric oxygen, oxygen toxicity, rat, ((brain slice) or (radio telemetry))

Search 2: hyperbaric oxygen, oxygen toxicity, seizure, aquaporin, gas channel, mouse

e. Results of Search:

Search 1: Searched in Projects,Publications and publication year limited between 1994 and 2019, Limit to:Project Abstracts, Admin IC: All, Fiscal Year: All Fiscal Years. Your search resulted in 0 projects. There were no relevant projects and publications found, even with altering the combination of words used in a search.

Search 2: Text Search: Search in Projects, Publications and publication year limited between 1994 and 2019, Limit to: Project Abstracts, Admin IC: All, Fiscal Year: Active Projects. Your search resulted in 0 projects. There were no relevant projects and publications found, even with altering the combination of words used in a search.

****Questions 4-10 refer to the Research Site and Protocol Principal Investigator.****

Information and/or documents required in questions 4-8 should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to BUMED by the Protocol Principal Investigator (PI) or institution staffs. If the Protocol PI prefers that BUMED staff contact the institution to obtain this information and/or documents, the PI must specifically request this action in writing. The Protocol PI should contact BUMED (see contact information on cover page) to arrange for submission of this written request.

4. Institutional Animal Care and Use Committee(s) (IACUC) Approval(s):

Institutional Animal Use Protocol Number:	IS00007438		
IACUC (initial) approval date:	December 13, 2019	Protocol expiration (rewrite) date:	December 10, 2022

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

USF MCOM Comparative Medicine is fully accredited by AAALAC International as program #000434, is managed in accordance with the Guide for the Care and Use of Laboratory Animals, the Animal Welfare Regulations, the PHS Policy, the FDA Good Laboratory Practices, and the IACUC Principles and Procedures of Animal Care and Use, has an assurance #D-16-00589 (A4100-01) on file with OLAW/PHS and maintains registration #58-R-0015 with USDA/APHIS/AC.

Comparative Medicine veterinarians are full-time staff, Florida-licensed, members of the AVMA, and are specialty board certified by ACLAM, and/or have research doctorates. Comparative Medicine maintains institutional membership with AALAS. All program staff are AALAS-certified, and many are either registered with the Society of Quality Assurance, and/or certified by the Academy of Surgical Research or are State of Florida-certified veterinary technicians.

Daily routine, weekend, holiday, and emergency animal care is provided by animal care program staff, all of whom are certified by the American Association for Laboratory Animal Science (AALAS), and more staff are certified at the highest, technologist level {LATG) than any other level. Cage changes occur twice weekly. Health surveillance occurs twice daily and assesses: food and water intake, fecal output, assurance of water supply and patency, and general appearance of animals (e.g. presence of lesions/injuries, coat condition, disposition). If at any time there is a medical emergency, the clinical veterinarian is notified immediately.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

[ ] Yes [ ] No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

[ ] Yes [X] No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

	Yes	**X**	No	Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Jay B. Dean (b) (6)

(Protocol Principal Investigator Printed Name) (Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

<u>BUMED Animal Use Appendix for Research Involving Animals</u>
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Grant/Contract PI Email: [redacted] **Phone:** [redacted]

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RS Attending Veterinarian: [redacted]

Attending Vet Email: [redacted] **Phone:** [redacted]

RS IACUC Point of Contact (POC): [redacted]

IACUC POC Email: [redacted] **Phone:** [redacted]

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Grants Office POC Email: [redacted] **Phone:** [redacted]

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or

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b. Date of Search: 16-17 December 2019

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d. Key Words and/or Search Strategy:

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Search 2: Text Search: Search in Projects, Publications and publication year limited between 1994 and 2019, Limit to: Project Abstracts, Admin IC: All, Fiscal Year: Active Projects. Your search resulted in 0 projects. There were no relevant projects and publications found, even with altering the combination of words used in a search.

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IACUC (initial) approval date:	December 13, 2019	Protocol expiration (rewrite) date:	December 10, 2022

5. Veterinary Care: Provide a brief description of the veterinary care plan at the research site. Describe routine care; weekend, holiday, and emergency care; and identify whether the attending veterinarian is on staff full-time or by contract.

USF MCOM Comparative Medicine is fully accredited by AAALAC International as program #000434, is managed in accordance with the Guide for the Care and Use of Laboratory Animals, the Animal Welfare Regulations, the PHS Policy, the FDA Good Laboratory Practices, and the IACUC Principles and Procedures of Animal Care and Use, has an assurance #D-16-00589 (A4100-01) on file with OLAW/PHS and maintains registration #58-R-0015 with USDA/APHIS/AC.

Comparative Medicine veterinarians are full-time staff, Florida-licensed, members of the AVMA, and are specialty board certified by ACLAM, and/or have research doctorates. Comparative Medicine maintains institutional membership with AALAS. All program staff are AALAS-certified, and many are either registered with the Society of Quality Assurance, and/or certified by the Academy of Surgical Research or are State of Florida-certified veterinary technicians.

Daily routine, weekend, holiday, and emergency animal care is provided by animal care program staff, all of whom are certified by the American Association for Laboratory Animal Science (AALAS), and more staff are certified at the highest, technologist level {LATG) than any other level. Cage changes occur twice weekly. Health surveillance occurs twice daily and assesses: food and water intake, fecal output, assurance of water supply and patency, and general appearance of animals (e.g. presence of lesions/injuries, coat condition, disposition). If at any time there is a medical emergency, the clinical veterinarian is notified immediately.

6. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither 6.a. nor 6.b. above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

7. Animal Procurement:

[ ] Yes [ ] No If the protocol involves Animal Welfare Act-regulated species, are the animals obtained legally from suppliers licensed by the USDA? If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met. If work is conducted outside the US, have animals been obtained legally in accordance with national policy? If wildlife are used, provide IACUC assurance that animals have been obtained legally and provide copies of applicable state, federal and/or international capture and use permits.

8. Overseas / Foreign Country Animal Work:

[ ] Yes [X] No Animal work will be performed outside the United States.

If "Yes," answer the following questions.

a. What is the law or regulation governing the use of animals in research in the research site's host country? Please provide a copy or link to this law or regulation in English.

b. Does the research site's host country adhere to European Union (EU) Directive 86/609 or EU Directive 2010/63 standards of animal housing and care?

c. If the research site is in Canada, does the institution hold a Canadian Council on Animal Care (CCAC) certificate?

d. Does the research site adhere to any national or international standards of animal housing and care that are more stringent than the host country's laws or regulations (such as AAALAC or CCAC)? If so, please describe below or provide a document, in English, that describes these standards.

e. Does the research site's host country or local institute require a local ethical committee review or Animal Care and Use Committee review? If so, please describe below or provide a document, in English, describing the committee's membership, purpose, authority and function.

9. Site Visits

	Yes	**X**	No	Does animal work involve at least one of the following species: dogs, cats, nonhuman primates, marine mammals? If yes, provide a planned start date for work in these species and point of contact for site visit coordination. Based on accreditation status, species used, and type of research conducted, a site visit to the performance site may be required.

10. Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the DoD Department of the Navy **Bureau of Medicine and Surgery** (BUMED) Veterinary Affairs Office prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Jay B. Dean (b) (6)

(Protocol Principal Investigator Printed Name) (Protocol Principal Investigator Signature and Date)

NOTE: In accordance with SECNAVINST 3900.38C, the DON BUMED Veterinary Affairs Officer (or designee thereof) will conduct a site visit to all sites using nonhuman primates, dogs, cats or marine mammals in the proposal, or where a site visit is deemed warranted.

Project Narrative

BAA Number: ONR BAA Number: N00014-20-S-B001

Title of Proposal: Mitigation of CNS oxygen toxicity: mitochondria-targeted antioxidant therapy and aquaporin gas channels

Prime Offeror: Office of Naval Research, Undersea Medicine Program

Technical Contact: (b) (6)(b) (6)(b) (6)(b) (6)
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Administrative/business contact:
(b) (6)

Proposed period of performance:
December 2019-October 2022

TECHNICAL APPROACH AND JUSTIFICATION

Overview of Project. The proposed 6.2 project focuses on the mitigation of central nervous system oxygen toxicity (CNS-OT) by targeting two, subcellular targets in the mammalian CNS that are thought to contribute to the pathogenesis of seizure genesis during exposure to hyperbaric oxygen (HBO_2): 1) mitochondrial superoxide ($\cdot O_2^-$) generation in the brain, and 2) O_2-/CO_2-permeabilities through aquaporin-4 (AQP4) at the blood-brain barrier (BBB).

Mitochondrial $\cdot O_2^-$ is generated at Complexes I-III of the electron transport chain (ETC) during cellular respiration [1-4]. We plan to significantly decrease $\cdot O_2^-$ production, and thus its reaction products downstream, during hyperoxia by mitochondrial-targeted antioxidant therapy (MitoTAT) using the supplement Mitoquinone-Q (MitoQ), which is already approved for human consumption.

Proposed experiments for Objectives 1-3 focus on MitoTAT and will be done in male Sprague-Dawley (SD) rats. Objective 4 will study AQP4, which is a gas channel that is permeable to CO_2 and presumably O_2, and thus postulated to function in CO_2 uptake during respiratory acidosis, metabolic CO_2 removal, and O_2 uptake at the BBB during exposure to HBO_2 [5, 6]. Objective 4 will be done in collaboration with (b) (6)(b) (6) at Case Western Reserve University (CWRU), Cleveland, Ohio. We plan to use AQP4-knockout (AQP4-KO) and wild-type (WT) mice, which are supplied by CWRU, to test the importance of AQP4 in O_2 & CO_2 uptake across the BBB during exposure to normocapnic & hypercapnic HBO_2 (i.e., HBO_2 $\pm CO_2$). The four research objectives are as follows:

Objective 1. Determine effects of HBO_2 ±MitoQ on the latency time to seizure (LSz) and the temporal pattern of neural activation across the brain stem, subcortical nuclei, hippocampus and motor cortices during the latent period that precedes initiation of visible motor/behavioral seizures.

Objective 2. Determine the effects of MitoQ during exposure to HBO_2 on the rate of production of reactive oxygen and nitrogen species (RONS) and mitochondrial membrane potential ($\Delta\Psi_m$) in the caudal Solitary Complex (cSC) of the medulla oblongata in rat brain tissue slices.

Objective 3. Determine the effects of MitoQ on neuronal electrophysiology during exposure to hyperoxia in cSC neurons in rat brain tissue slices.

Objective 4. Determine the role of AQP4 in the pathogenesis of CNS-OT by testing the effects of HBO_2 $\pm CO_2$ on the LSz in mice with decreased AQP4 expression (AQP4-KO mice [5, 6]) and increased AQP4 expression (WT mice in which AQP4 is upregulated by oxidative preconditioning (OxPC) [7]).

Chapter One

1. Larsen, F.J., et al., *Regulation of mitochondrial function and energetics by reactive oxygen species.* Free Radical Biology and Medicine, 2012. **53**: p. 1919-1928.
2. Turrens, J.F., *Superoxide production by the mitochondrial respiratory chain.* Bioscience reports, 1997. **17**(1): p. 3-8.
3. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* Journal of Physiology, 2003. **552.2**: p. 335-344.

4. Ciarlone, G.E. and J.B. Dean, *Acute hypercapnic hyperoxia stimulates reactive species production in the caudal solitary complex of rat brain slices but does not induce oxidative stress.* American Journal of Cell Physiology, 2016b. **311**: p. C1027-C1039.
5. Nagelhus, E.A. and O.P. Ottersen, *Physiological roles of aquaporin-4 in brain.* Physiological Reviews, 2013. **93**: p. 1543-1562.
6. Xu, K., et al., *Increased cerebral capillary density in Aquaporin-4 knockout mouse.* FASEB Journal, 2016. **30**: p. 721.9.
7. Cevik, N.G., et al., *The effects of hyperbaric air and hyperbaric oxygen on blood-brain barrier integrity in rats.* Brain Research, 2013. **1531**: p. 113-121.