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TITLE: Efficacy of Oxygen-Carrying Therapeutic and Antioxidant Drug in Treatment of Cerebrovascular Complications as a Consequence of Severe Blast TBI

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CONTRACTING ORGANIZATION: Naval Medical Research Center, Silver Spring, MD

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14. ABSTRACT

Blast-induced traumatic brain injuries (bTBI) are of a significant concern among the US warfighters with a longer lasting impact on their quality of life. In spite of clinical advances that improved survivability of service members with severe head injuries, there is a lack of effective treatments that prevent delayed brain damage caused by the initial blast exposure. Our previous studies on impact head injury have shown a 50 % reduction in brain tissue oxygenation and restoration of oxygenation to near pre-injury levels after treatment with an oxygen-carrying fluid (NVX-108. This study aims at utilizing NVX-108 in preventing blast-induced deficits in brain tissue oxygenation in gyrencephalic (ferrets) animal model. Utilization of an oxygen-carrying substance, NVX-108, may potentially contribute to an increase in oxidative stress. For this reason, the current study will use an antioxidant, N-acetylcysteine, NAC, in conjunction with NVX-108 to assess the efficacy of this combination treatment in preventing blast-related deficits in the brain tissue oxygenation and the associated damage to the structure and function of cerebral vasculature. In the period covered by this report, institutional and ACURO approvals were obtained for the animal use protocol, equipment was upgraded/purchased and/or calibrated for the measurement of brain tissue oxygen tension, intracranial pressure, and cerebral blood flow. Equipment was also fabricated and set up for the exposure of ferrets to blast wave. Staff was trained on surgical methods. Despite delays caused by Covid-19 pandemic significant progress was made which will enable us to carry out the work planned on this study. Details of the delays and the plans to resolve them are provided in the report.

15. SUBJECT TERMS
Ferret, blast traumatic brain injury, brain tissue oxygenation, oxidative stress, brain tissue oxygen tension, N-acetylcysteine, dodecafluoropentane (NVX-108), cerebral blood flow, intracranial pressure, blood-brain barrier, cerebral microcirculation, hypoxia, cerebral vascular dysfunction, oxygen therapeutics, antioxidant, intravital microscopy

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1. INTRODUCTION:

A frequent cause of brain injuries on the battlefield to US Warfighters is exposure to explosive blast shock waves, which in severe cases can result in both penetrating and non-penetrating brain injury. In spite of clinical advances that improved survivability of service members with severe head injuries, there is a lack of effective treatments that prevent delayed brain damage caused by the initial exposure to blast. We have previously shown that traumatic brain injury due to a head impact leads to up to 50% reduction in brain tissue oxygenation, which was restored to near pre-injury levels with treatment with an injectable oxygen-carrying fluid (NVX-108) that is currently in clinical trials in the US for use to treat stroke and certain types of brain cancer. Delivering such a product shortly after injury in combat could prevent the effects of blood vessel injury by providing oxygen to brain tissue and preventing further tissue damage. In the current study, we will use ferrets as a blast injury model because of their gyrencephalic brain structure, which permits a better approximation of the human condition than lissencephalic rodent models. We will expose ferrets to high-intensity blast in an air-driven shock tube to characterize the extent and timing of brain tissue oxygenation deficits in order to construct a picture of the injury. We will then assess the ability of a therapeutic intervention combining the oxygen therapeutic NVX-108 and the antioxidant NAC to prevent the post-injury deficits. A concern with the use of oxygen-carrying fluids is increase in pathological tissue oxidation attributed to increase in oxygen levels and, therefore, the use of these products in conjunction with an antioxidant may be a more effective treatment strategy. Overall, our mission is to provide data demonstrating the injurious effects of exposure to blast on oxygen levels and blood flow in the brain and the effectiveness of a promising oxygen carrying drug and anti-oxidants as a translatable treatment for blast-related brain damage sustained by service members of the US Military. Such an intervention has the potential to prevent life-long disability and improve the quality of life of military service members wounded in combat.

2. KEYWORDS:

Ferret, blast traumatic brain injury (bTBI), traumatic brain injury (TBI), brain tissue oxygenation, oxidative stress, brain tissue oxygen tension (PbtO2), N-acetylcysteine (NAC), dodecafluoropentane (NVX-108), cerebral blood flow (CBF), intracranial pressure (ICP), blood-brain barrier (BBB), cerebral microcirculation, hypoxia, cerebral vascular dysfunction, oxygen therapeutics, antioxidant, intravital microscopy, cerebral vascular reactivity.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim/task	Timeline	NMRC	WRNMMC	% completion	Completion date
Specific Aim 1: Evaluate the effect of severe bTBI on functional and pathological outcomes by monitoring measures of cerebral function ($P_{bt}O_2$, CBF, ICP, and oxidative stress).					
Major Task 1 - Assess the effects severe bTBI on vascular function 2h, 3 and 14 days post-BOP.					
Subtask 1: Write and submit animal protocol to NMRC IACUC for approval followed by submission to ACURO for review.	1	Dr. Ahlers	CAPT Bell	100%	Jun 2020, Oct 2020, and Dec 2020
Subtask 2: Procure signal conditioning unit for data acquisition for ICP monitoring	1	Dr. Ahlers		100%	June 2020
Milestone # 1: *IACUC and ACURO approvals obtained for animal use protocol and signal conditioning unit for ICP data acquisition purchased and installed.*	1-4	Dr. Ahlers		100%	Dec 10, 2020
Subtask 3: Have pre-start up meeting with NMRC Veterinary Medicine staff and begin ordering sets of ferrets staggered to achieve a rate of 4 animal experiments per month.	4	Dr. Ahlers		100%	Jan 7, 2021
Subtask 4: Expose ferrets to single BOP exposure (19 psi) and allow animals to recover until the appropriate study time points (2h, and 3 and 14 days post-BOP). Groups will be randomized.	4-8	Dr. Ahlers			
Subtask 5: Assess $P_{bt}O_2$, CBF, ICP, and other physiological parameters *in vivo* at appropriate study time points after BOP exposure.	4-8	Dr. Ahlers			
Subtask 6: Euthanize all animals at end points of 2h, or 3 or 14 days post-BOP and collect brains for oxidative stress assessments and histopathology studies.	4-8	Dr. Ahlers			
Milestone # 2: Report data for characterization of severe bTBI effects on vascular function using $P_{bt}O_2$, CBF, and ICP, as surrogates of vascular function over time without a drug intervention.	4-8	Dr. Ahlers	CAPT Bell		
Major Task 2 - Assess the effects of severe bTBI (19 psi BOP exposure) on oxidative stress and histopathological markers.					
Subtask 1: One hemisphere of the brains collected in Subtask 6 under Major task 1 above (the right hemisphere), will be used for histopathological assessment of severe bTBI on oxidative stress and hypoxia markers.	8-11	Dr. Ahlers			
Subtask 2: The left hemisphere of the brains collected in Subtask 6 under Major task 1 will be used for molecular assessments of oxidative stress and hypoxia.	8-11	Dr. Ahlers			
Milestone # 3: Report data for characterization of BOP-induced effects on oxidative stress markers and histopathology.	11-12	Dr. Ahlers			

Specific Aim 2 - Assess the therapeutic efficacy of an oxygen therapeutic PFC dodecafluoropentane (NVX-108) and antioxidant NAC in mitigating blast-induced decrements in functional and pathological outcomes.					
Major Task 1 - Efficacy testing of NVX-108 on vascular function after severe bTBI.					
Subtask 1: Procure pharmaceutical grade NVX-108 from NuvOx Pharma, Tuscan, AZ.	12	Dr. Ahlers			
Subtask 2: Continue ordering new sets of animals, staggered in 6 treatment groups at a rate of 3-4 animals per month. A total of 30 animals are planned for year 2.	12	Dr. Ahlers			
Subtask 3: Expose ferrets to single BOP (19 psi) and allow animals to recover until the appropriate study time points (2h, and 3 and 14 days post-BOP exposure). Animals will be randomized to the study groups.	12-19	Dr. Ahlers			
Subtask 4: Administer NVX-108 after BOP intravenously (2 doses of 0.5-1 ml/kg, 90 minutes apart) as a therapeutic intervention.	12-19	Dr. Ahlers			
Subtask 5: Assess $P_{bt}O_2$, CBF, ICP, and other physiological parameters *in vivo* 2h, 3 days and 14 days post-blast.	12-19	Dr. Ahlers			
Subtask 6: Euthanize all animals at end points of 2h, or 3 or 14 days post-blast and collect brains for oxidative stress and histopathology assessments.	12-19	Dr. Ahlers			
Milestone # 4: Report data for efficacy of treatment with NVX-108 alone on vascular function using $P_{bt}O_2$, CBF, and ICP, as surrogates of vascular function over time.	19-20	Dr. Ahlers	CAPT Bell		
Major Task 2 - Assess the efficacy of NVX-108 in mitigation of severe bTBI effects on oxidative stress and hypoxia markers.					
Subtask 1: One hemisphere of the brains collected from animals treated with NVX-108 (the right hemisphere), will be used for histopathological assessment of severe bTBI effects on oxidative stress and hypoxia markers.	20-23	Dr. Ahlers			
Subtask 2: The left hemisphere of the brains collected from animals treated with NVX-108 will be used for molecular assessments of oxidative stress and hypoxia.	20-23	Dr. Ahlers			
Milestone # 5: Report data for efficacy of treatment with NVX-108 to mitigate of blast overpressure- induced effects on oxidative stress markers and histopathology.	20-23	Dr. Ahlers	CAPT Bell		
Major Task 3 - Efficacy testing of a therapy combining NVX-108 with NAC on vascular function after severe bTBI.					
Subtask 1: Procure pharmaceutical grade IV-injectable NAC (Acetadote) from Cumberland Pharmaceuticals, Inc.	24	Dr. Ahlers			

Subtask 2: Continue ordering new sets of animals, staggered in groups at a rate of 4 ferrets per month for a total of 30 ferrets in year 3.	24	Dr. Ahlers			
Subtask 3: Expose ferrets to single BOP exposure (19 psi) and allow animals to recover until the study time points of 2h, 3, or 14 days post-BOP exposure. Animals will be randomized to the study groups.	24-31	Dr. Ahlers			
Subtask 4: Administer NVX-108 and NAC after BOP intravenously (2 doses of 0.5-1 ml/kg nvx-108 and 150 mg/kg NAC, 90 minutes apart) as a therapeutic intervention.	24-31	Dr. Ahlers			
Subtask 5: Assess $P_{bt}O_2$, CBF, ICP, and other physiological parameters *in vivo* 2h, 3 days and 14 days post-blast	24-31	Dr. Ahlers			
Subtask 6: Euthanize all animals at end points of 2h, or 3 or 14 days post-blast and collect brains for oxidative stress assessments and histopathology.	24-31	Dr. Ahlers			
Milestone # 6: Report data for efficacy of treatment of severe bTBI with a combination of NVX-108 and NAC on vascular function using $P_{bt}O_2$, CBF, and ICP, as surrogates of vascular function over time.	24-31	Dr. Ahlers	CAPT Bell		
Major Task 4 - Efficacy testing of a therapy combining NVX-108 with NAC on oxidative stress and hypoxia markers after severe bTBI					
Subtask 1: One hemisphere of the brains collected from animals treated with NVX-108 and NAC (the right hemisphere), will be used for will be used for histopathological assessment of severe bTBI effects on oxidative stress and hypoxia markers.	31-34	Dr. Ahlers			
Subtask 2: The left hemisphere of brains collected from animals treated with NVX-108 and NAC will be used for molecular studies.	31-34	Dr. Ahlers			
Milestone # 7: Report data for efficacy of severe bTBI with a combination of NVX-108 and NAC to mitigate BOP- induced effects on oxidative stress markers and histopathology.	31-34	Dr. Ahlers			
Milestone #8: Prepare final report for entire study and manuscripts for publication in peer-reviewed journals.	34-36	Dr. Ahlers	CAPT Bell		

What was accomplished under these goals?

Major activities:

Under specific aim # 1, the following was accomplished:

1) Approval of animal care and use protocol by WRAIR/NMRC IACUC on Oct 13, 2020
2) ACURO approval was granted in Dec, 2020
3) Protocol initiation meeting with members of the veterinary medical staff was held to authorize starting animal studies.
4) The equipment for the measurement of brain tissue oxygenation, using phosphorescence quenching method, was reconfigured and updated by the manufacturer.
5) A ferret holding device for blast exposure of ferrets in our blast tube has been fabricated at NMRC. Anesthesia system for pre-blast anesthesia of ferrets was set up.
6) Research assistants are being trained on surgical and experimental procedures in training animals to optimize all steps prior to conducting full experiment in pilot animals as described in our IACUC- and ACURO approved protocol.
7) Two pilot animals have been purchased and will be used in June 2021.
8) 12 animals have been ordered from Marshall BioResources and will be used in September when they reach the age (24-weeks) at which time they can be used on our IACUC-approved protocol.
9) Submission of an amendment to the IACUC- approved protocol to request approval for using animal vendors other than Marshall BioResources

Specific objectives and outcomes:

The objectives of the first year of the project were to purchase or fabricate the equipment necessary for obtaining the physiological parameters detailed in the study, i.e., intracranial pressure (ICP) and brain oxygen tension ($P_{bt}O_2$) using the phosphorescence quenching method. In addition, we sought to train staff members on all procedures needed to perform experiments. The trainings include ferret handling, anesthesia induction, exposing animal to blast overpressure in the NMRC/WRAIR air-driven shock tube, vascular access, implanting ICP catheters, drilling craniotomies for measuring ($P_{bt}O_2$) using the phosphorescence quenching method and collecting cerebral blood flow, and harvesting brain measurements. Finally, we had planned to complete 2-4 pilot studies in ferrets and begin data collection. We have been able to accomplish procurement of equipment or equipment parts as well as provide training to staff for performing procedures. However, the unanticipated shutdown of all animal work at NMRC due to the COVID19 pandemic in 2020, the subsequent delays we faced in the reopening phases imposed by social distancing measures and building occupancy caps, and finally the shortage in ferret availability caused by the exponential increase in ferret use for COVID-related research, we have not been able to complete the pilot studies and begin data collection. In spite of the significant delays, we have been able to procure two ferrets for pilot studies, which we plan on performing in June 2021. In addition, we purchased animals which will be available for us to use in the end of September 2021. We anticipate to be able to start collecting data in earnest within that timeframe.

What opportunities for training and professional development has the project provided?

A post-doctoral fellow, three research assistants and veterinary personnel involved with this project received training to learn and optimize all surgical and animal handling techniques, which led to acquisition of new skills and knowledge about the cerebrovascular effects of traumatic brain injury. In addition, activities on this project have created an opportunity for staff members to mentor younger investigators and, thus, acquire experience as mentors.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We plan on using two ferrets to complete two pilot studies that involve exposing the animals to all procedures described on the protocol. Specifically, we plan on exposing one animal to sham procedures and the other to blast with control fluid administration. The data collected from these animals will be used to optimize data acquisition (if needed) and will be included in the final data analyses, if no unanticipated events occur.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The study is likely to make an impact on the field of traumatic brain injury research. The data from this study will show whether exposure to blast overpressure results in reduction of brain tissue oxygenation and whether that and other known deficits caused by blast-related neurotrauma, including disruption of the blood brain-barrier and cerebral blood flow, can be ameliorated with treatment with an antioxidant combined with a promising oxygen-carrying therapeutic.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

One of our key personnel, CAPT Goforth, was assigned to support COVID19 research at the beginning of the pandemic and is no longer with our program. Dr. Ahlers has incurred the additional % effort at no additional cost to see through the remaining goals of the projects.

Actual or anticipated problems or delays and actions or plans to resolve them

Delays were experienced in receiving IACUC approvals due to the evolving nature of Covid-19 pandemic. This has delayed the initiation of animal studies. We are working with our Command veterinary staff to minimize any further delays. In addition, there has been a significant increase in the demand for the use of ferrets in research in general and for COVID19 research in specific since the beginning of the COVID19 pandemic and initial submission of the animal protocol. The only ferret vendor that has been used by our department, Marshall BioResources, has been overwhelmed by the increased demand for ferrets and the supply is falling short of the demand. We are unable to start our study, which requires 24 week-old and at least 1 kg ferrets, due to this shortage in ferrets from the supplier. This shortage is predicted to last for months before the supply chain recovers. Since we have not performed any surgeries/experiments yet, at this juncture, we can easily change the vendor and acquire animal from a different source. The COVID19 pandemic has already caused delays in this project and we hope to avoid further delays due to unavailability of animals. Adding the new vendor is an opportunity for us to start the experiments in earnest and avoid more delays.

The following steps have been taken to address this unexpected problem:

1) We have purchased twelve (12) 7-week old ferrets in April, which will be 24-weeks old in September 2021. This has secured animals of the appropriate age and weight for us to use for experiments planned in September.
2) We will use two animals we currently have in house as pilot animals, as described in our IACUC and ACURO protocol.
3) We submitted an amendment to the IACUC requesting approval of additional vendors for ordering ferrets to avoid further delays

Changes that had a significant impact on expenditures

We have not received FY21 funds in the amount of $275k for this project as of May 2021. Not receiving these funds will impact our ability to continue our work on this project and meet the remaining study goals.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

NMRC
Name: Dr. Stephen T Ahlers
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Dr. Ahlers has provided oversight and participated in developing animal use protocol.

Name: Dr. Rania Abutarboush
Project Role: Associate Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Dr. Abutarboush took the lead in developing animal use protocol; collaborated with the manufacturer on refurbishing our brain tissue oxygen monitoring system; trained research assistants on surgical techniques and experimental procedures in rodents which will be used in the ferret pilot animals; and procured animals.

Name: Dr. Usmah Kawoos
Project Role: Associate Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Dr. Kawoos has participated in developing animal use protocol; collaborated on design and fabrication of a holding device and setting up of an anesthesia system to be used for exposure of ferrets to blast overpressure in our blast tube; trained research assistants on surgical techniques and experimental procedures in rodents which will be used in the ferret pilot animals.

Name: Carl Goforth
Project Role: Associate Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 0
Contribution to Project: CAPT Goforth was assigned to support COVID19 research at the beginning of the pandemic and is no longer with our program. Dr. Ahlers has incurred the additional % effort at no additional cost to see through the remaining goals of the projects.

Name: Eileen Reed
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: provide assistance to all research activities and train on experiment performance.

Name: Cameron Watson
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: provide assistance to all research activities and train on experiment performance.

Name: Kapinga Ngalula
Project Role: Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: provide assistance to all research activities and train on experiment performance.

USU:
Name: Randy Bell, MD
Project Role: PI
Nearest person month worked: 0
Contribution to Project: Principal Investigator

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: