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TITLE: Assessing the Effects of a Novel Ketone Ester in an Established Rodent Model of Blast Traumatic Brain Injury

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

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None

14. ABSTRACT

Blast-induced traumatic brain injury (TBI) is a concern in the military population, with many personnel exposed to blast from improvised explosive devices. At the cellular level TBI has been shown to decrease energy production, create oxidative products that are associated with destructive processes and lead to cell death. Because of these findings, many researchers think TBI is at least partially a result of dysfunction of brain energy metabolism. In this study, animals were given a novel ketone ester after a blast exposure. In the hours and days following the exposure we expected this intervention to accelerate recovery and even prevent secondary injury processes. We have tested this hypothesis by monitoring blood glucose and ketone levels, body weight, behavioral responses, and oxidative stress for two weeks after exposure to blast in animals administered a ketone ester versus controlled conditions. Overall, while two weeks of ester administration resulted in a state of ketogenisis, most behavioral outcomes were unaffected. However, there was a trend suggesting that the ketone ester ameliorated blast-induced oxidative stress. The results are presented in detail in this report.

15. SUBJECT TERMS
TBI, ketone ester, glycolysis, metabolism, mitochondria, oxidative stress, neurological function, blast injury, brain, cortical, neurotrauma, locomotion, motor, cognitive performance, rodents

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1. Introduction

Blast-induced traumatic brain injury (TBI) is a concern in the military population, with many personnel exposed to blast from improvised explosive devices. At the cellular level, TBI has been shown to decrease energy production, create oxidative products that are associated with destructive processes, and lead to cell death. Because of these findings many researchers think TBI is at least partially a result of dysfunction of brain mitochondria, the structures in cells which are responsible for a large proportion of energy produced in the cell. In this study, animals were given a novel ketone ester after exposure to blast. In the hours and days following, we expected that that this intervention would accelerate recovery and even prevent secondary injury processes. We tested our hypothesis by administering ketone ester for two weeks following the blast, then collecting tissue and testing for pathology and repair in the brain. Assessments involved examination of behavior, blood biochemistry, and brain tissue biochemistry. Due to unexpected pandemic-related delays during the course of the study the specific aims were not executed as originally proposed by assessing acute (4 hr and 24 hr post-injury) and chronic (14 days post-injury) effects of diet conditioning with the ketone ester. Based on the findings from the assessment of potential benefits in utilizing the ketone ester for 14 days post-injury our focus was shifted on carrying out a comprehensive assessments for the chronic time point after injury. This revised plan was devised in consultation with the Science Officer.

2. Keywords

TBI, ketone ester, glycolysis, metabolism, mitochondria, oxidative stress, neurological function, blast injury, brain, cortical, neurotrauma, locomotion, motor, cognitive performance, rodents

3. Accomplishments

- **What were the major goals of the project?**
    - **Specific Aim 1:** To demonstrate the effects of novel ketone ester [R,S-1,3-butanediol acetoacetate diester (BD- AcAc2)] administration on mitochondrial and neurological function following blast injury at acute (4 and 24 hour) time points.
        - Major Task 1: IACUC and ACURO Approval (1 Month)- *Completed*
        - Major Task 2: Perform Animal Experiments – 4 hour and 24 hour Cohorts (2-12 Months)- *Revised*

Our initial assessment showed blast-related oxidative stress 14 days after blast exposure (under specific aim 2) and a potential benefit of utilizing the novel ketone ester in ameliorating the stress. In consultation with the Science Officer an alteration to our research strategy was agreed upon which allowed for utilizing the remaining resources on this study towards focusing on performing comprehensive analysis of blast-induced oxidative stress, inflammation and neurodegeneration and the effect of the administration of ketone ester on possibly improving the outcome.

- **Specific Aim 2:** To measure the chronic effects of BD- AcAc2 administration on mitochondrial and neurologic function 2 weeks following blast-induced injury.
  - Major Task 2: Perform Animal Experiments – 2 week Cohort (2-12 Months) *Completed*

- **What was accomplished under these goals?**
  - Major task 1 was completed. We obtained local IACUC approval on March 31, 2020 and ACURO Approval on May 1, 2020. Protocol initiation meeting was conducted on June 24, 2020. Animal experiments were started in August 2020.
  - Animal experiments were carried out to complete the work under specific aim 2 and lab assays to evaluate oxidative stress were completed. Results are presented here.

    Blood analysis was conducted for glucose and ketone levels before and after exposure to blast. Immediately after blast exposure ester diet (80% standard diet + 20% BD- AcAc2, ketone ester) or standard diet was administered via oral gavage and the standard chow was replaced by either ester diet or standard diet which was provided ad libitum. The body weight of animals was monitored. The animals were trained on rotarod and Morris water maze tests on three consecutive days pre-blast and were tested again 14 days after blast exposures and diet treatment to evaluate the efficacy of ketone ester in ameliorating blast-related deficits in motor function and memory and spatial learning. On the same day animals were euthanized to collect biospecimens for assessing oxidative stress via ELISAs and immunohistochemistry. To summarize the results:

    1) Blood analysis showed no effect of blast on glucose and ketone levels when blast exposed animals were compared with diet-matched sham animals. Animals on ester diet showed increase in ketone levels and decrease in glucose levels from their pre-diet conditioning

baseline and the levels returned back to the baseline by day 2 (for glucose) and day 8 (for ketones). Preliminary results show that administration of ester diet may be beneficial in restoring glucose and ketone levels to pre-blast levels and may have a potential role in circumventing energy deficit caused by blast exposure.

2) Assessment of motor function (rotarod test) and spatial learning (Morris water maze test) did not show significant effects of blast or diet conditions on the performance on these tests. Briefly, the animals were trained on three consecutive days prior to blast exposure and were tested on day 14 post-blast. Figure 1. shows average time spent on the spinning rod, average speed, best time, and best speed presented as percentage change from the pre-blast training baseline. Various parameters on Morris water maze test including trials average time, average speed, distance travelled, number of target zone entries, latency to first platform entry, time spent in target zone, and number of platform crossings were measured. For the sake of brevity only two parameters (number of target zone entries and latency to first platform entry are presented in Figure 2. On the Y-maze spontaneous alternation test, which was conducted 14 days post-blast and assesses short-term and spatial memory, animals on ester diet spent less time being immobile and also showed an increase in the number of alterations and moves, thus showing beneficial role of ketone ester in maintaining short-term memory and encouraging exploratory behavior. Figure 3 presents the results of Y-maze test.

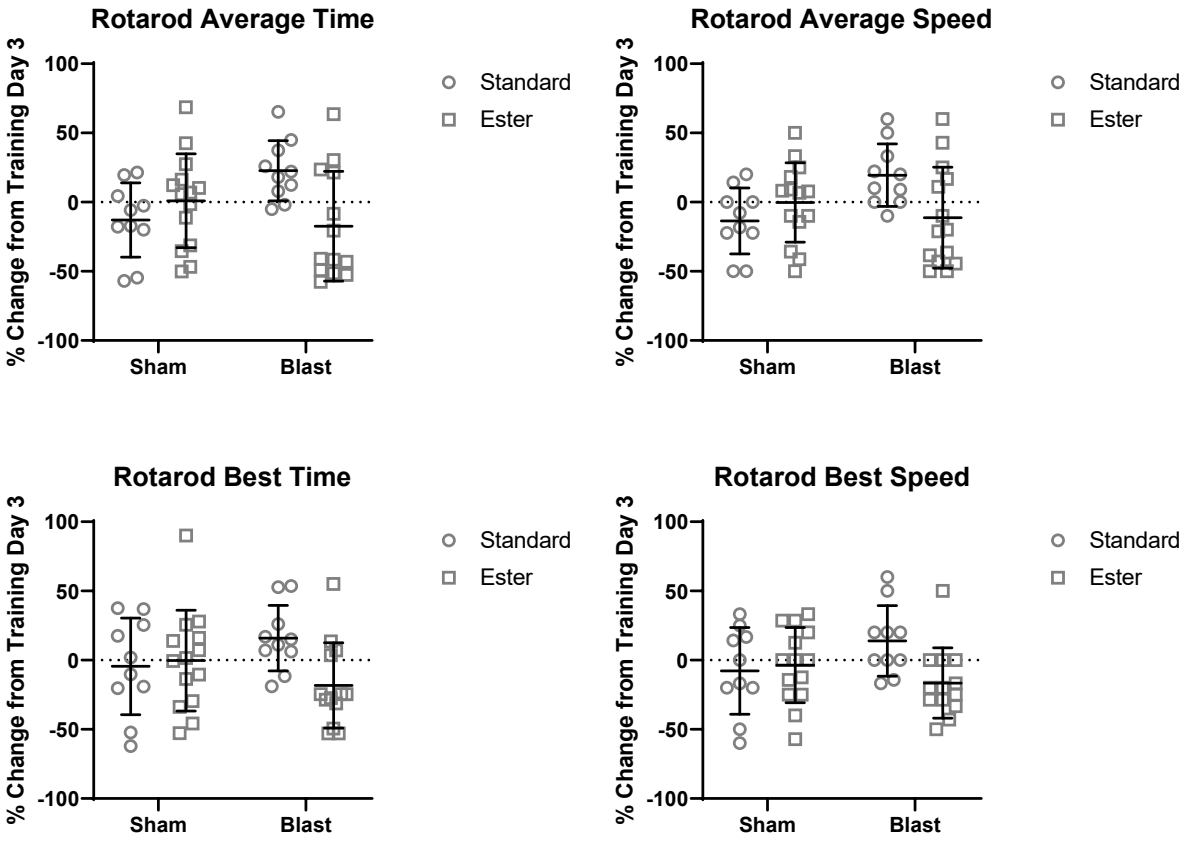

Figure 1. Performance of animals on rotarod test. No significant differences were observed among groups on all measures of the test.

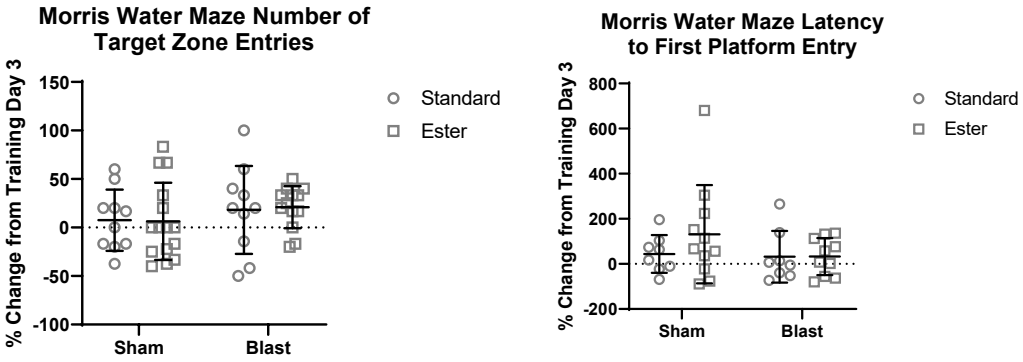

Figure 2. Performance of animals on Morris water maze test. No significant effect of diet or blast condition was observed among groups on all measures of the test.

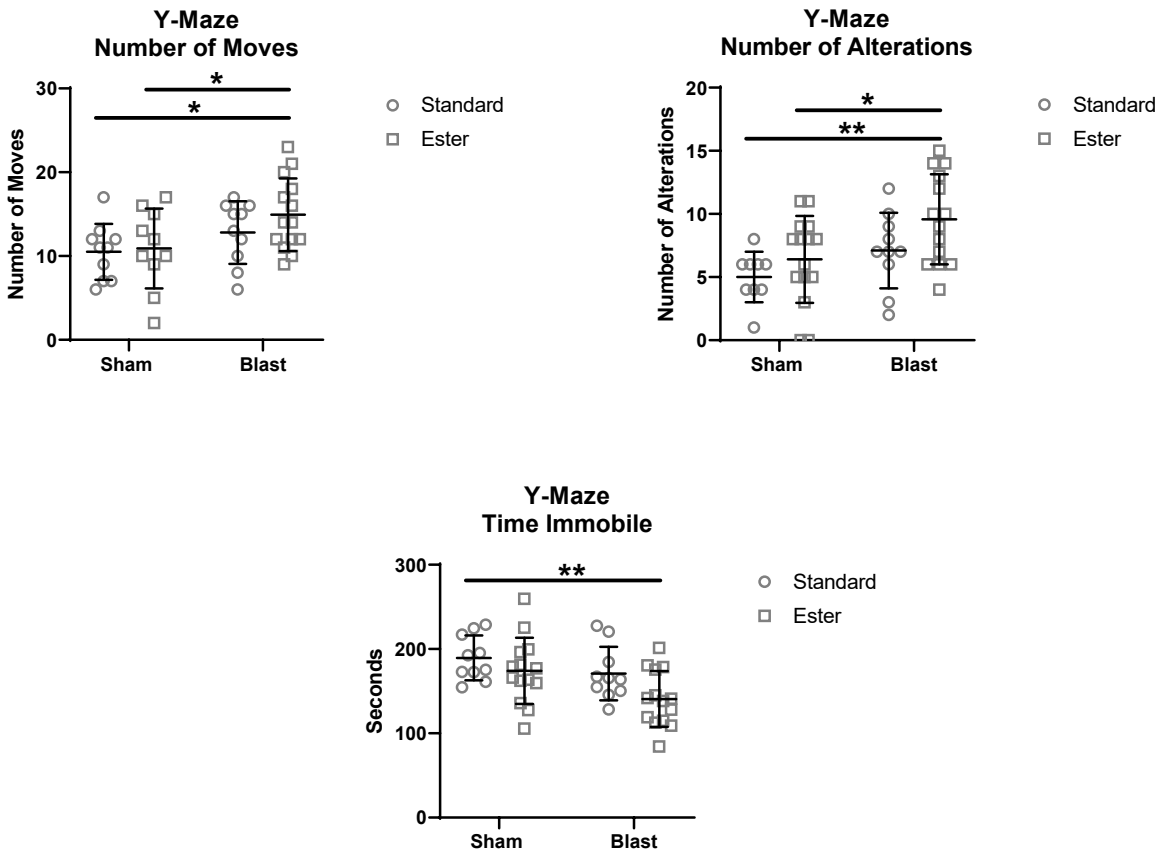

Figure 3. Results of Y-maze test. Significant effect of ketone ester was observed in improving the performance on this test. Data was analyzed by two-way ANOVA followed by Tukey's post-hoc analysis. * $p<0.05$, ** $p<0.01$

3) The evaluation of oxidative stress induced by blast-exposure and potential benefit of ketone ester in improving the outcome was done by conducting assays specifically for quantifying the amount of reactive oxygen species (ROS), the ratio of reduced and oxidized glutathione (GSH/GSSG), and total antioxidant capacity (TAC) in frontal cortex and hippocampus of the brain. A significant decrease in the ROS in hippocampus was seen in ester-sham group when compared to the standard diet-sham condition and a trend towards reduction in ROS was observed in ester-blast vs. standard-blast group (Figure 4). A similar trend was seen in GSH/GSSG ratio in frontal cortex and no effect was observed in hippocampus (Figure 5). At resting state a high GSH/GSSG ratio is expected which is reduced in response to oxidative stress. The TAC assay probed for small molecule and protein antioxidants including uric acid, ascorbate, albumin, transferrin etc., There was a significant increase in TAC after administration of ester diet indicating an enhanced ability to neutralize oxidative stress (Figure 6). Evaluation of an oxidative stress marker, 3-nitrotyrosine (3NT) was conducted using immunohistochemistry (IHC) techniques. A significant increase in oxidative stress as observed by an

increase in 3NT expression was seen in standard-blast group and administration of ester diet was associated in a significant reduction in the expression of 3NT in ester-blast group. Figure 7 shows IHC evaluation of 3NT.

4) Additionally, a significant increase in inducible nitric oxide synthase, iNOS, labeled cells evaluated by IHC, was observed in cortex in standard-blast group which was near standard-sham levels in both ester-sham and ester-blast groups. NOS regulates the production of nitric oxide, which is considered a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations.

5) IHC evaluation of blast-related neuronal degeneration by Fluoro-Jade staining showed a significant increase in degenerating neurons in cortex in standard-blast group when compared to standard-sham and a significant reduction in the expression of Fluoro-Jade in ester-blast when compared to ester-blast.

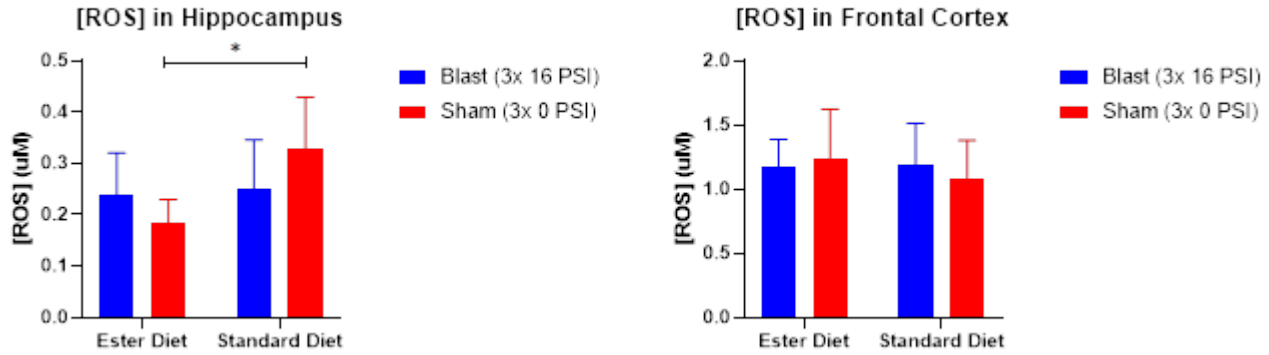

Figure 4.Blast-related oxidative stress indicated by levels of ROS and the effect of ester diet in reducing the stress. Data analyzed by two-way ANOVA followed by Dunnett's post-hoc test. * p<0.01

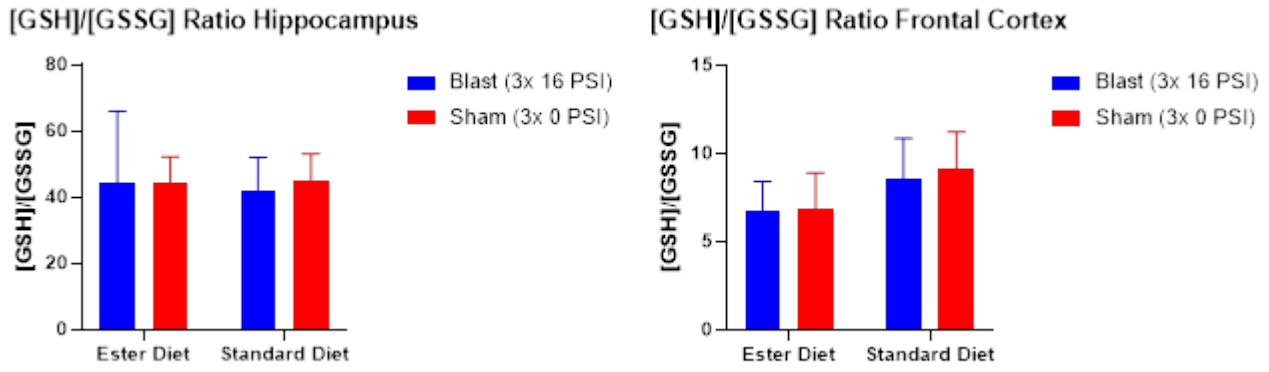

Figure 5.Effect of blast and ester diet on glutathione presented as the ratio of reduced (GSH) and oxidized (GSSG) glutathione.

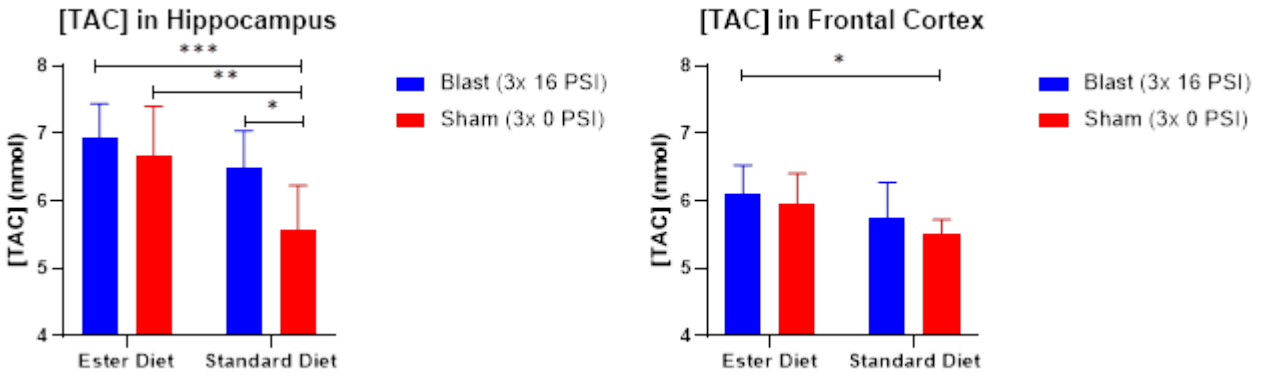

Figure 6. Effect of blast and ester diet on total antioxidant capacity (TAC). A significant increase in TAC under ester diet conditions are indicative of the ability to defend against oxidative stress. Data analyzed by two-way ANOVA followed by Dunnett's post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Taken together, the data supports the potential of ketone ester in ameliorating blast-induced oxidative stress, inflammatory response, and neuronal degeneration.

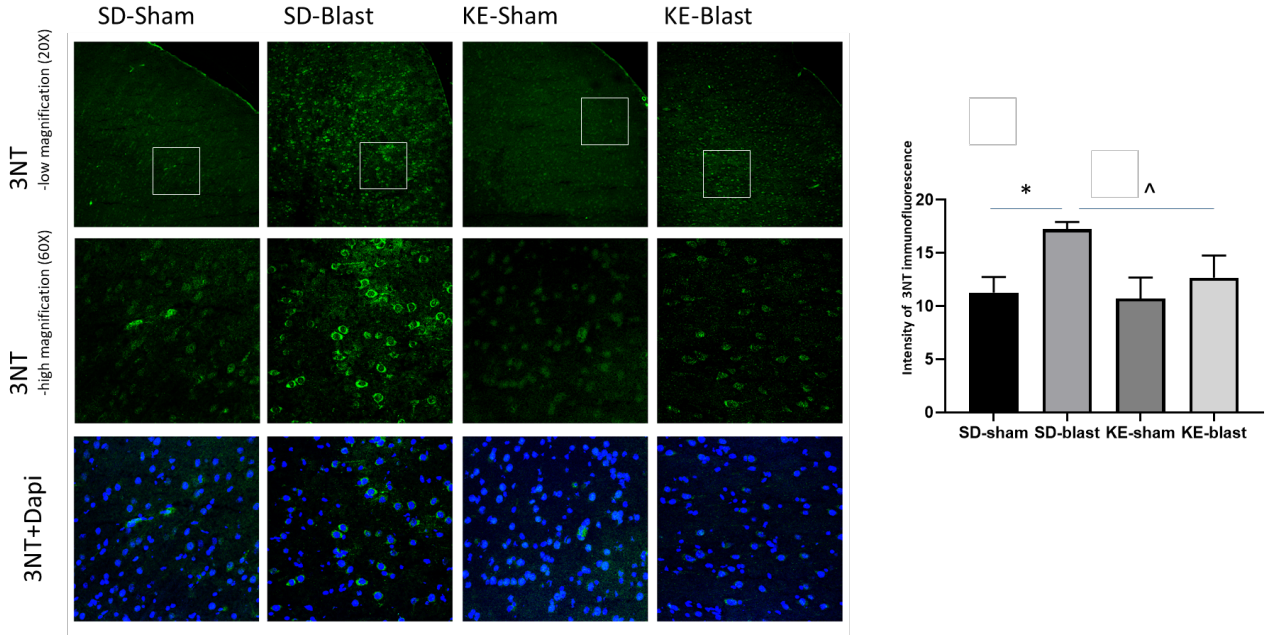

Figure 7 The expression of 3-nitrotyrosine (3NT) in different groups. SD-Blast (standard-blast) presented higher levels of 3NT and KE-Blast (ester-blast) showed a significant reduction in the expression of 3NT when compared to SD-Sham (standard-sham). Data analyzed by 2-way ANOVA followed by Dunnett's post-hoc analysis. ^ $p<0.05$, * $p<0.01$.

- **What opportunities for training and professional development has the project provided?**
    - This project provided training and professional development of a research associate, post-doctoral fellow, and two research assistants.
- **How were the results disseminated to communities of interest?**
    - An abstract based on this work was accepted for poster presentation at 2022 National Neurotrauma Symposium. This abstract will be indexed in the Journal of Neurotrauma.

- We have a manuscript in preparation.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Not applicable.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**
  - We were invited to submit a pre-proposal for FY22 PRMRP expansion award based on the findings of this study. The pre-proposal was submitted in May 2022.
- **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

- **Changes in approach and reasons for change**
  - The approach was modified to conduct more comprehensive assessments under specific aim 2 and not conduct the tasks under specific aim 1. Due to unanticipated delays related to Covid 19 pandemic, there was a significant delay in the initiation of animal work. The limitations on the maximum number of personnel who were able to be onsite during the pandemic led to our decision of starting the study with aim 2 (14 day cohort). Aim 1 involved acute time points with a number of procedures and assessments to be conducted in animal experiments in a short period of time (4 hr or 24 hr post-blast), which would require more personnel to be on-site for conducting animal work. However this was not feasible during 2020, given the uncertainties associated with the pandemic. During the course of the study our findings from aim 2 showed promising results which formed the basis for conducting comprehensive assessment of the efficacy of ester diet in improving outcomes related to oxidative stress, inflammation, and neurodegeneration after exposure to blast. The PI and the science officer agreed upon utilizing the remaining resources on the project in continuing our focus on aim 2 of the study and

perform various assessments of the efficacy of ketone ester in ameliorating blast-induced deficits.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
    - Nothing to report
- **Changes that had a significant impact on expenditures**
    - Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - Nothing to report
- **Significant changes in use or care of human subjects**
    - Nothing to report
- **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**
        - An abstract titled, 'Efficacy of ketone ester diet in improving outcome after blast-induced traumatic brain injury' was accepted and will be published in the Journal of Neurotrauma.
        - A manuscript is in preparation
    - **Books or other non-periodical, one-time publications.**
        - Nothing to report
    - **Other publications, conference papers, and presentations.**
        - A poster will be presented at 2022 National Neurotrauma Symposium in Atlanta.
- **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?**

The following individuals have worked on this project over the course of this study:

Name:	Dr. Stephen T Ahlers
Project Role:	Principal investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Project oversight and planning
Funding Support:	PRMRP/CDMRP
Name:	LT Claire Modica
Project Role:	Associate investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Preparation of IACUC protocol and planning
Funding Support:	PRMRP/CDMRP
Name:	Usmah Kawoos
Project Role:	Senior Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Planning and execution of the study
Funding Support:	PRMRP/CDMRP

Name:	Dr. Ye Chen
Project Role:	Senior Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Supervised planning and conduction of lab assays
Funding Support:	PRMRP/CDMRP
Name:	Dr. Ming Gu
Project Role:	Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Participated in animal experiments and conducted immunohistochemistry evaluation
Funding Support:	PRMRP/CDMRP
Name:	Mr. Jonathan Statz
Project Role:	Research associate/Biostatistician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Participated in planning and conducting of animal experiments, data collection, and analysis.
Funding Support:	PRMRP/CDMRP
Name:	Mr. Jacob Patterson
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	Participated in planning and conducting of animal experiments, data collection, lab assays, data analysis and presentation.
Nearest person month worked:	10

Contribution to Project:	Participated in planning and conducting of animal experiments, data collection, lab assays, data analysis and presentation.
Funding Support:	PRMRP/CDMRP

- **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**
    - Nothing to report
- **QUAD CHARTS**
    - Nothing to report

9. Appendices

- None