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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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14. ABSTRACT

Blast-induced traumatic brain injury (bTBI) among the service personnel has been of increasing concern due to their operational risk of being exposed to blast events. The pathophysiology of bTBI at the cellular level may be exacerbated due to high energy demand and the subsequent decrease in energy production followed by accumulation of free radicals leading to oxidative stress. The energy power house (mitochondria) uses acetyl-Co-A whose production primarily relies on the breakdown of glucose, which is an oxygen dependent process. This process may be hindered during injury making in unable to meet the high energy demands. Alternate energy substrates like ketone bodies are more readily available for breakdown and production of acetyl-Co-A and may be an efficient source of energy for meeting the high energy demands. To study the efficacy of utilizing a ketone ester (KE) diet in our model of bTBI, rats were exposed to three 110 kPa blasts with 0.5 h interval between blasts and were assigned to one of the four blast/diet conditions (sham+KE, blast+KE, sham+standard diet (SD), blast+SD). The diet assignment was initiated immediately after exposure to blast/sham conditions and the diet was made available ad libitum for 14 days. During the course of 14 days the blood glucose and ketone levels along with body weight were monitored and compared with pre-blast/sham levels. Animals were also trained on rotatod (motor function) and Morris Water Maze (spatial memory) prior to exposure and were evaluated again on day 14 post-exposure. Additionally, working memory was tested using Y-maze on day 14 after the exposure. The effect of different condition on oxidative stress are currently being evaluated by performing assays and immunohistochemistry methods on brain tissue to probe for oxidative stress markers and antioxidants. 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 KE 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). The data shows no significant differences among groups on behavior and rotarod tasks. An increase in oxidative stress marker (3NT) was observed in blast+SD group and a significant reduction in 3NT expression was seen in blast+KE in comparison to blast+SD. Preliminary results suggest that administration of KE 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. KE diet may also be beneficial in alleviating blast-induced oxidative stress.

15. SUBJECT TERMS
Novel ketone ester diet, blast overpressure, traumatic brain injury, motor function, cognitive performance, oxidative stress.

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1. Introduction

Blast-induced traumatic brain injury (TBI) is an increasingly prevalent health 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 (Giza, 2014). 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 will be given ketone ester after a blast exposure. In the hours and days following, we expect that that this intervention will accelerate recovery and even prevent secondary injury processes. We will test the first part of our hypothesis by administering ketone ester immediately after blast, allowing mitochondrial processes to function for several hours, then collecting tissue and testing for mitochondrial function. We will test the second part of our hypothesis by continuing to administer the ketone ester for two weeks following the blast, then collecting tissue and testing for pathology and repair in the brain. Assessments will involve examination of behavior, blood biochemistry, and brain tissue biochemistry.

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)
    Major Task 2: Perform Animal Experiments – 4 hour and 24 hour Cohorts (2-12 Months)
  - **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 1: Perform Animal Experiments – 2 week Cohort (2-12 Months)

- **What was accomplished under these goals?**

Specific Aim 1:
Major task 1 under 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 begun in August 2020. However work was conducted at a slower pace due to reduced on-site staffing in compliance with Covid-related guidelines.

Specific Aim 2:
Animal experiments under major task 1 were completed (subtask 1). Lab assays and immunohistochemistry methods were used to determine oxidative stress (subtask 2). Data analysis and graphing from the outcomes of subtasks 1 and 2 were completed.

To study the efficacy of utilizing a ketone ester (KE) diet in our model of bTBI, rats were exposed to three 110 kPa blasts with 0.5 h interval between blasts and were assigned to one of the four blast/diet conditions (sham+KE, blast+KE, sham+standard diet (SD), blast+SD). The diet assignment was initiated immediately after exposure to blast/sham conditions and the diet was made available ad libitum for 14 days. Blood analysis was conducted for glucose and ketone levels before and after exposure to blast. Immediately after blast exposure ester diet (standard diet + 20% BD- AcAc2, KE) or standard diet (SD) was administered via oral gavage and the standard chow was replaced by either KE or SD which was provided *ad libitum*. The body weight of animals was also monitored. The results are presented in Figure 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). There was no effect of diet on the rate of increase in body weight.

The animals were trained on rotarod (Figure 2) and Morris water maze (Figure 3) tests on three consecutive days pre-blast/sham exposure and were tested again 14 days after blast exposures and diet treatment to evaluate the efficacy of ester diet in ameliorating blast-related deficits in motor function, memory and spatial learning. On day 14 post-exposure, the animals were also tested on Y-maze (Figure 4) for evaluating their working memory, following which the animals were euthanized to collect biospecimens for assessing oxidative stress via lab assays (ROS/RNS, GSH/GSSG, and total antioxidant capacity) and immunohistochemistry. Lab assays are currently underway. The results from immunohistochemistry of 3NT, an oxidative stress marker are presented in Figure 5. Briefly, exposure to blast led to an increase in the expression of 3NT indicative of an increase in oxidative stress ($p<0.01$, blast+SD vs. sham+SD). The blast+KE group showed a significant reduction in 3NT ($p <0.05$, blast+KE vs. blast+SD).

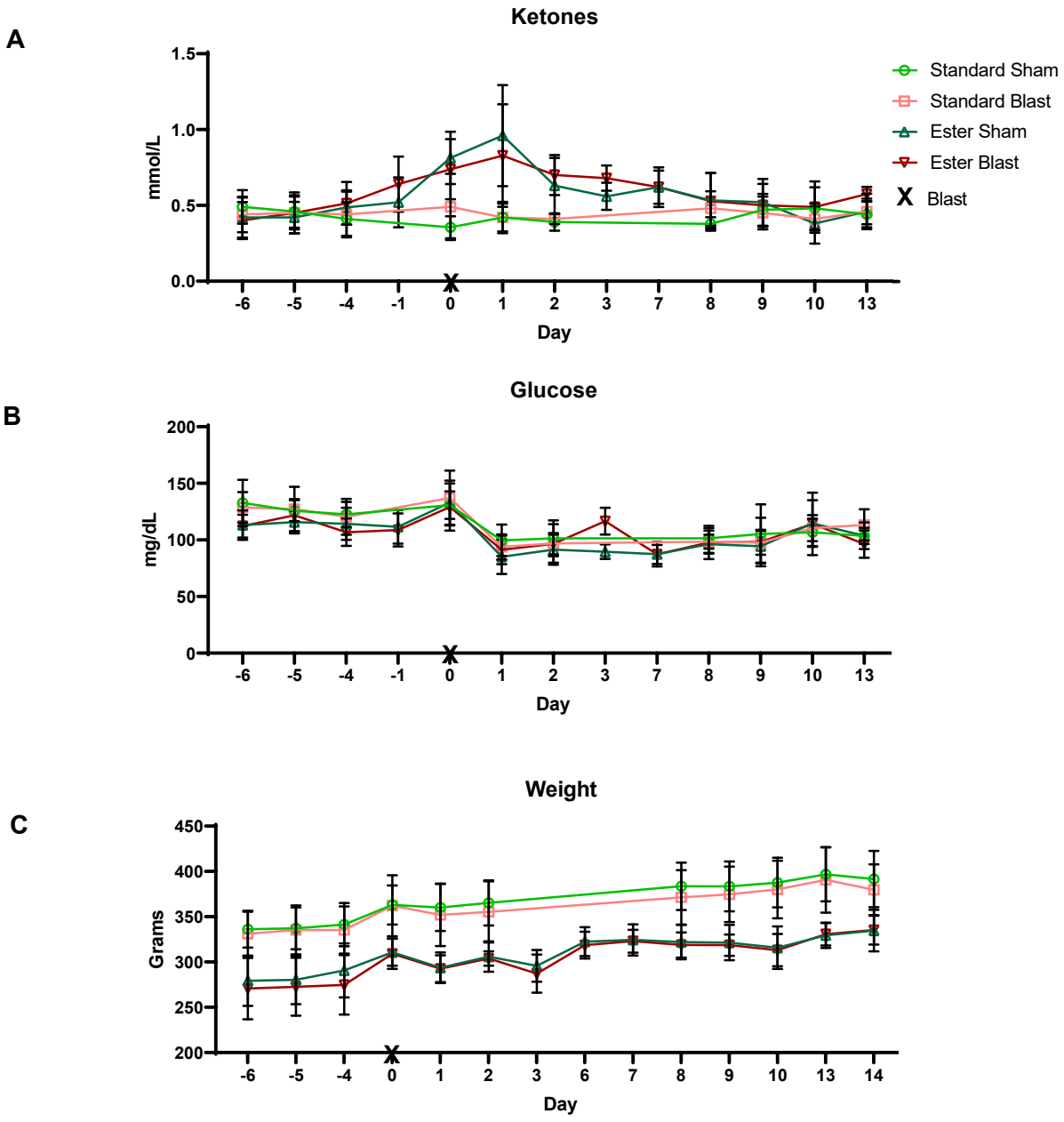

Figure 1. Changes in blood ketone and glucose levels and body weight over time. A) Increase in ketone levels were seen in groups on ester diet. B) Initial decrease in glucose levels were seen in all groups and there were no inter-group differences. C) There was no effect of diet on the rate of increase in body weight. Note that the differences in the starting weights between SD and KE cohorts are due to delaying of SD cohorts by one week because of inclement weather.

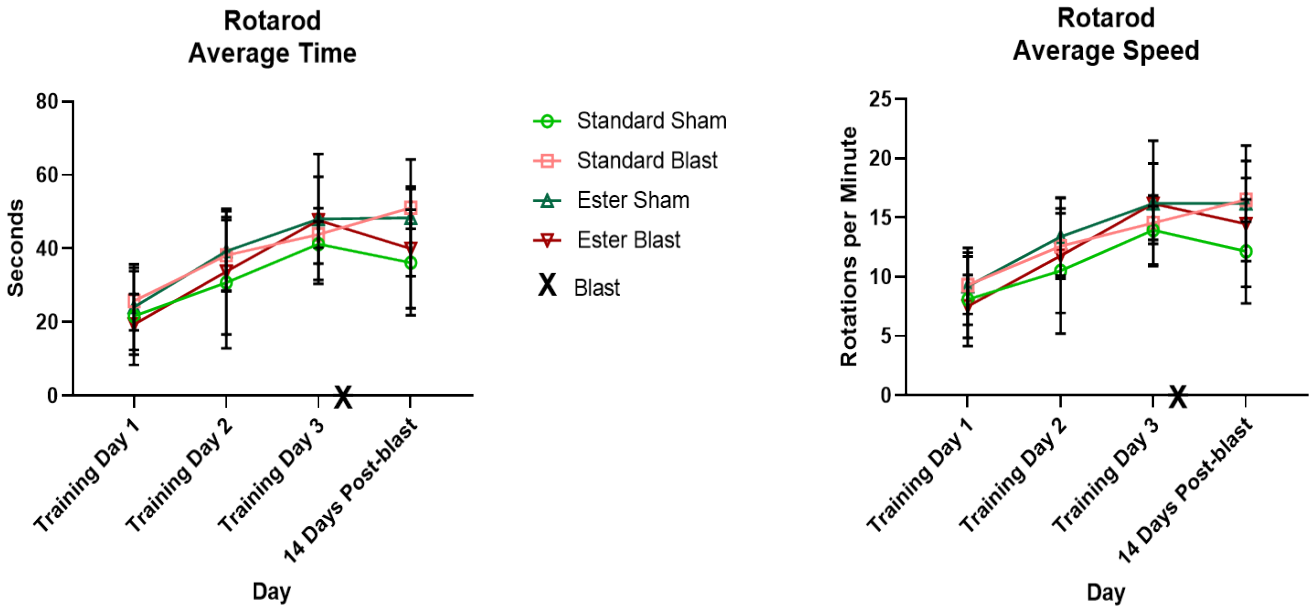

Figure 2. Performance on rotarod test to assess motor function. No-blast related deficits in rotarod performance were observed and no significant differences among groups were seen.

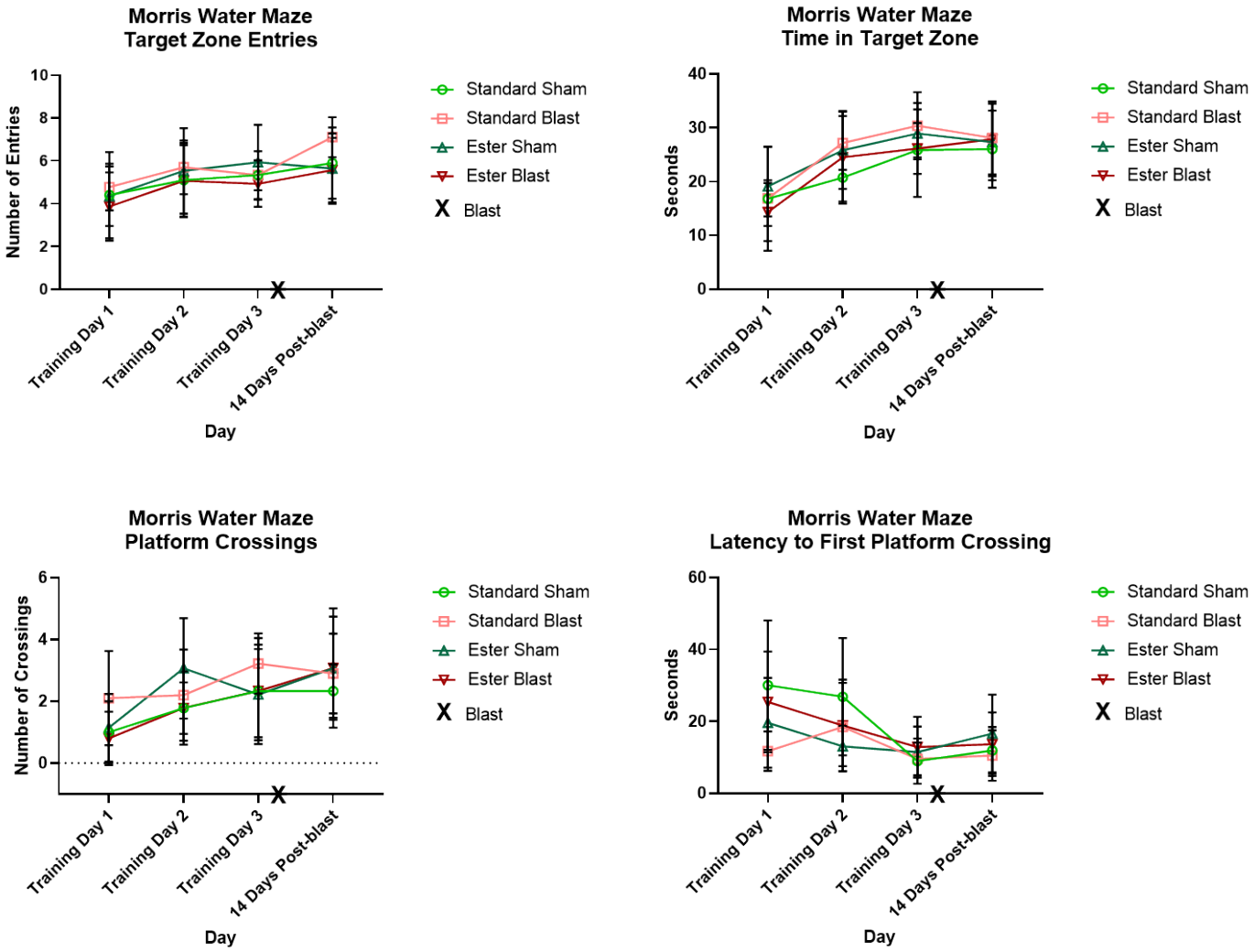

Figure 3. Spatial reference memory test using Morris Water Maze. There were no effects of blast or diet condition on different parameters in Morris Water Maze test.

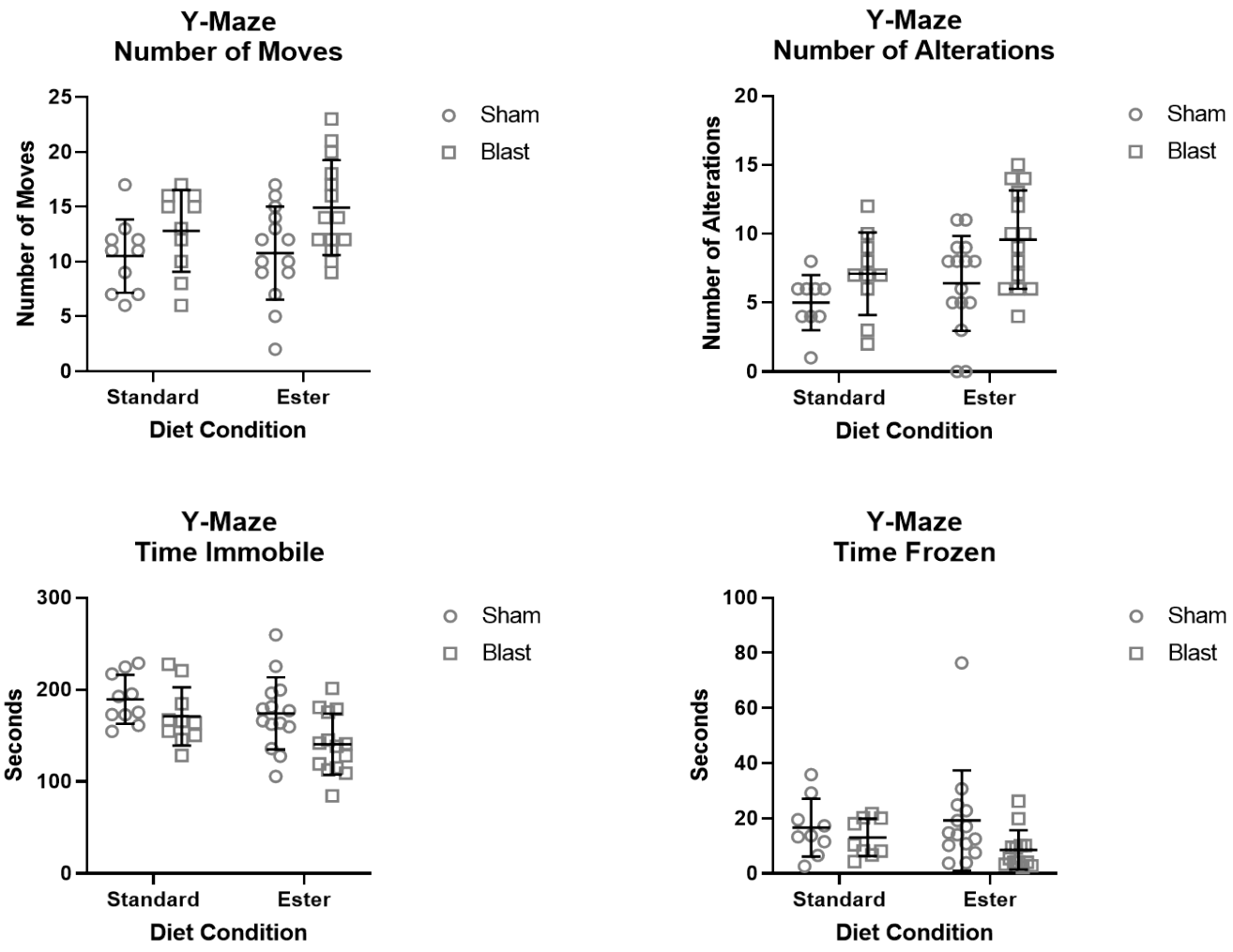

Figure 4. Y-maze test for working memory. Blast+SD and blast+KE conditions presented an upward trend in number of alterations and number of moves and a downward trend in time immobile and time frozen. However the differences were not statistically significant.

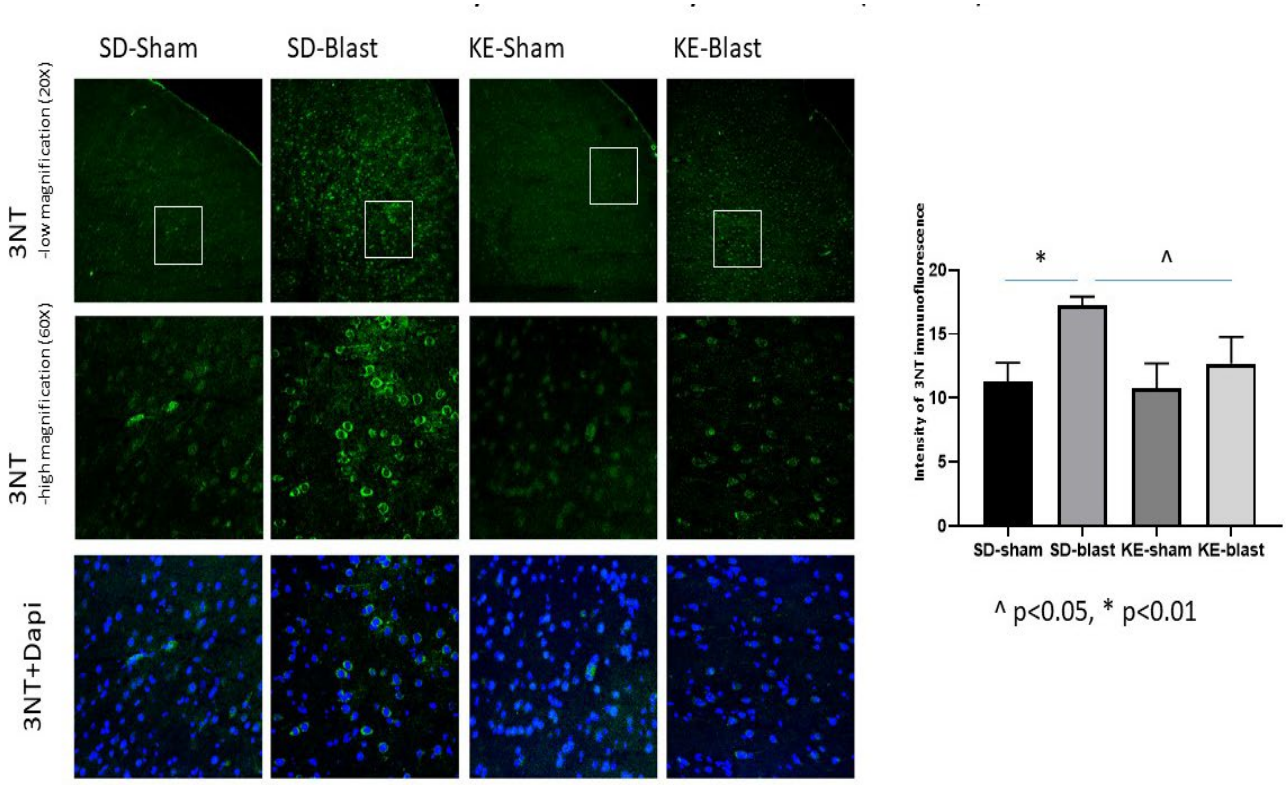

Figure 1. The expression of 3-nitrotyrosine (3NT) in different groups. SD+Blast presented higher levels of 3NT and KE+Blast showed a significant reduction in the expression of 3NT when compared to SD+Sham. Data analyzed by 2-way ANOVA followed by Dunnett's post-hoc analysis.

Preliminary results show that administration of KE diet may be beneficial in restoring glucose and ketone levels to pre-blast levels under chronic conditions (14 days) and may have a potential role in circumventing energy deficit caused by blast exposure. KE diet also has an effect on alleviating oxidative stress. Further experiments and analyses are planned to assess the effect of blast on oxidative stress and the potential benefit of administering KE diet in preventing or reducing oxidative stress in the chronic phase.

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

This project has provided training and professional development to a post-doctoral fellow. It is also providing an opportunity for professional development of our research associate and research assistants.

- **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?**

No cost extension of this study was approved. We plan on conducting experiments to accomplish goals under major task 2 of specific aim 1 (i.e., assessment in the acute phase after blast). Animal experiments will be followed by lab assays to investigate any potential benefits of KE diet administration on oxidative stress induced by blast exposure.

4. Impact

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

Nothing to report

- **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**

Nothing to report

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

Delays in getting started with animal work. Due to evolving COVID-19 situation, there were unanticipated delays in getting the animal protocol approval and the mandatory protocol initiation meeting conducted. A virtual mandatory protocol meeting was held which allowed us to proceed with the conduction of animal work. Some animal experiments were conducted and lab assays are were run to determine the state of blast-induced oxidative stress and any potential benefits of administering the ester diet. Further assays are being conducted to continue the evaluation of oxidative stress and the potential role of ester diet in ameliorating the stress and improving the outcome.

- **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.**

  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. Participating and other Collaborating Organizations

- **What individuals have worked on the project?**

Name	Dr. Stephen T Ahlers (No change)
Name	Dr. Usmah Kawoos
Project Role	Associate Investigator
Research Identifier	N/A
Nearest person month worked	4
Contributions to project	Assisted with project oversight, planning of experiments and assays, and data analysis
Funding support	N/A
Name	Dr. Ye Chen
Project Role	Associate Investigator
Research Identifier	N/A
Nearest person month worked	2
Contributions to project	Planning and guiding experiments and assays
Funding support	N/A
Name	Keegan Statz
Project Role	Research Associate
Research Identifier	N/A
Nearest person month worked	3
Contributions to project	Execution of animal experiments and data analysis
Funding support	N/A
Name	Jacob Patterson
Project Role	Research Assistant
Research Identifier	N/A
Nearest person month worked	3

Contributions to project	Execution of animal experiments, lab assays and data analysis
Funding support	N/A

- **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

Nothing to report

9. Appendices

Nothing to report