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TITLE: **Probing the mechanistic role of vascular dysfunction and vascular inflammation in TBI-mediated cognitive dysfunction**

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CONTRACTING ORGANIZATION: **Carl T. Hayden Medical Research Foundation**

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14. ABSTRACT
Traumatic brain injury (TBI) is a major cause of mortality/morbidity among service-members/veterans and is linked to long-term development of aging related dementia disorders through still poorly-defined mechanisms. We are testing the hypothesis that an important etiopathologic basis of TBI-related cognitive dysfunction is cerebrovascular dysfunction and vascular inflammation resulting in chronic brain hypoperfusion. We are also testing the hypothesis that TBI confers susceptibility to later development of cardiovascular risk factor (specifically diabetes/hyperglycemia)-related cerebrovascular dysfunction leading to cognitive impairment. In Aim 1 we will measure the cognitive function of Sprague-Dawley rats exposed to TBI by fluid percussion injury and determine the relationship with cerebrovascular function (in vivo by MRI and ex vivo by circle of Willis artery vasoreactivity) and vascular inflammation. In Aim 2 we will determine whether TBI and diabetes-related metabolic derangements or β-amyloid confer synergistic deleterious effects on cognitive function, cerebrovascular function and inflammation. We completed the rat cohorts which underwent TBI or sham operation and measured in-vivo and ex-vivo cerebrovascular function data. Our data so far show impaired cognitive function at 3 and 6 months following TBI with some regional association between cognitive and in vivo cerebrovascular function and modest reduction in pial arterial smooth muscle-dependent function post-TBI. Induction of diabetes using streptozotocin did not lead to greater cognitive impairment in TBI rats.

15. SUBJECT TERMS
Traumatic brain injury, cognitive dysfunction, endothelial function, dementia, inflammation

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TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Traumatic brain injury (TBI) is a major cause of mortality and morbidity among service-members and veterans and has been linked to long-term development of aging related dementia disorders through still poorly-defined mechanisms. We are testing the hypothesis that an important etiopathologic basis of TBI-related cognitive dysfunction is through cerebrovascular dysfunction and vascular inflammation resulting in chronic brain hypoperfusion. We are also testing the hypothesis that TBI confers susceptibility to later development of cardiovascular risk factor (specifically diabetes/hyperglycemia)-related cerebrovascular dysfunction leading to cognitive impairment. In Aim 1 we will measure the cognitive function of Sprague-Dawley rats exposed to diffuse TBI by fluid percussion injury and determine the relationship with cerebrovascular function (in vivo by MRI and ex vivo by circle of Willis artery vasoreactivity) and vascular inflammation. In Aim 2 we will determine whether TBI and diabetes-related metabolic derangements or β-amyloid confer synergistic deleterious effects on cognitive function, cerebrovascular function and inflammation.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Traumatic brain injury, cognitive dysfunction, endothelial function, dementia, inflammation, cerebrovascular disease, vascular imaging

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Please see attachment.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Obtain institution and DOD approval for live animal work.
Accomplishment: Institutional and DOD approvals for live animal work were obtained during the first few months of the funding period.

2. Compare 180d in vivo cerebral blood flow and reactivity by MRI and ex-vivo by circle of Willis arteries between TBI vs. uninjured rats and determine the relationship of vascular function with measures of cognitive function and degree of neuropathology.
Subtask 1: Produce cohorts of uninjured and TBI rats (n=6 each)
Subtask 2: Draw blood and conduct cognitive testing at 3 and 6 months post injury

Accomplishments: We have completed the cohorts of sham and TBI rats in terms of cognitive behavioral testing (3 and 6 months), in-vivo MRI vascular perfusion testing and ex-vivo vasoreactivity testing. Serologic testing of all samples is scheduled for batch testing.
Identified challenges: Meticulous attention to scheduling and animal handoffs represented the greatest challenge since the project involves complex procedures performed at 3 institutions (University of Arizona College of Medicine-Phoenix, Barrow Neurological Institute, and Phoenix VA). Communication between the sites has been the greatest challenge, where regular in person meetings, clear email, and a shared project calendar system have been rewarded with continued success. This part of the project (animal cohort testing) was accomplished prior to COVID-19 onset.
Scientific Findings:
Cognitive function was assessed using 3 standardized measures: novel object recognition (NOR), novel object location (NOL) and temporal order object recognition (TOR), which represent assessments of short-term, long-term, and working memory, respectively. The discrimination ratio represents the ratio of attention to the familiar versus novel object, where a value of 0.5 indicate chance performance. The 3 and 6-month data show impairment in all 3 cognitive domains at 3 months

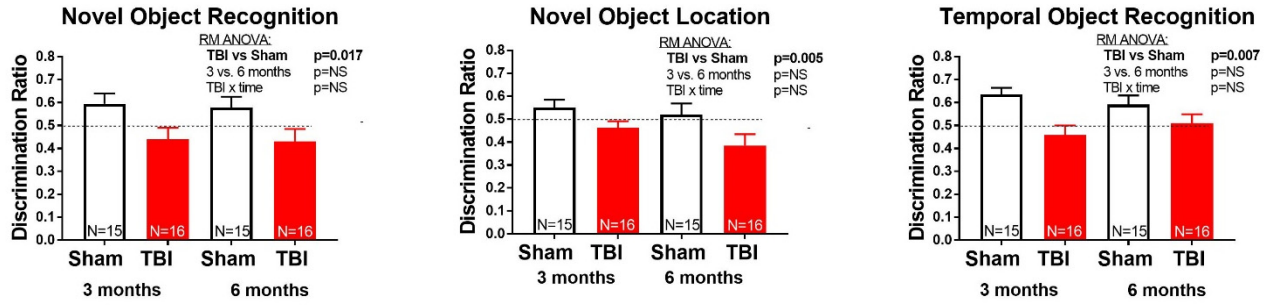

Fig. 1. Cognitive function in rats at 3 and 6 months after mild diffuse TBI show impairment in novel object recognition (short term memory), novel object location (long term memory) and temporal order object recognition (working memory) at 3 months and persisting at 6 months post injury (repeated measures ANOVA).

that persist up to 6 months (Fig. 1). This is consistent with our hypothesis that **diffuse brain injury results in sustained, chronic cognitive dysfunction**. This finding of cognitive dysfunction 6 months following mild-moderate TBI in this rat model enhances the value of this experimental animal model in recapitulating what has been observed in human epidemiologic studies.

Subtask 3: Conduct in vivo cerebral blood flow and cerebrovascular vasoreactivity using MRI in brain injured and uninjured rats.
Accomplishments: We have completed imaging of all rat cohorts with TBI and sham injury including those with LPS pretreatment and those followed by streptozotocin injection.
Identified challenges: In our last annual report, we indicated that after observing attenuated dilator response to hypercapnea in different brain regions in the initial cohorts of uninjured and TBI rats (following labor-intensive analyses after imaging procedure), Dr. Quarles conducted an extensive investigation and confirmed a technical error in calculation of flow rates being used leading to

exposure of rats to ~1% CO_2 instead of the planned 5% CO_2 needed to perform hypercapneic experiments. This error did not affect baseline cerebral blood volume measurements of sham versus TBI rats. This error was corrected in rat cohorts undergoing sham or TBI pretreated with LPS, or treated with STZ.

Scientific Findings: We observed blood volume differences in both right and left medial hippocampus (Fig. 2), with similar trend in lateral hippocampus. Looking at other regions, non-significant trend of increased resting blood volume in TBI versus sham was also seen in almost all regions (Fig. 3). Consistently, the in vivo blood volume results were equivalent between the left and right hemisphere, which validates the diffuse and bilateral aspects of the employed experimental model. The finding of increased blood volume is contrary to our hypothesis and needs further elucidation. This difference, noted in vivo, as well as observations of modest impairment in smooth muscle-dependent dilation in ex-vivo pial artery in mTBI rats (discussed below), suggest persistent cerebrovascular perturbations 6 months following mTBI. Furthermore, we noted significant inverse relationship between resting hippocampal blood volume and long term memory cognitive performance (NOL) (Fig. 4) that shows association between cerebrovascular and cognitive function.

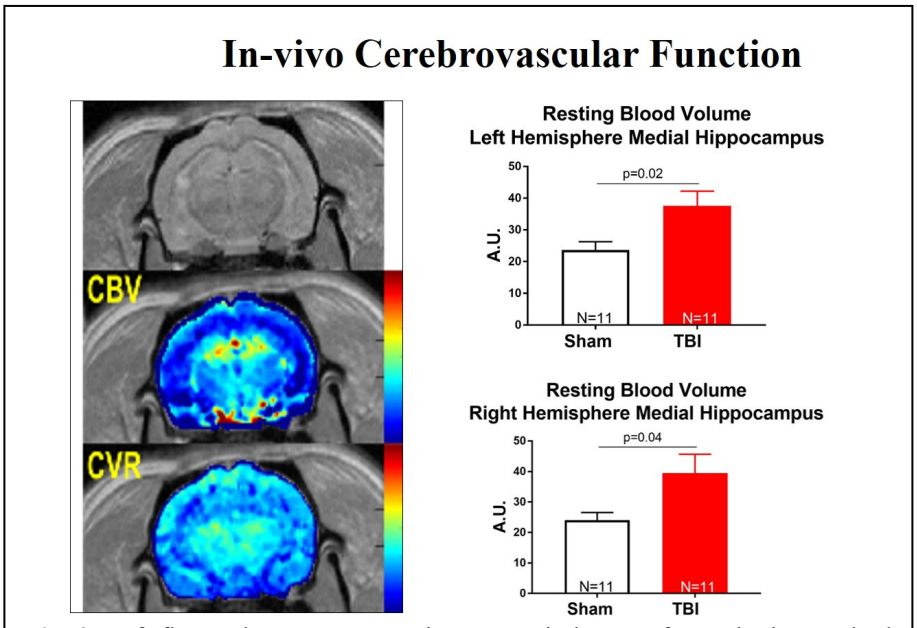

Fig. 2. Left figure shows representative anatomic image of a rat brain, cerebral blood volume (CBV) and hypercapneic vasoreactivity (CVR) quantification in our cohort. There was regional variation in resting blood volume. Contrary to our hypothesis, TBI resulted in increased resting blood volume in both left and right medial hippocampus (and trend towards same results in lateral hippocampus).

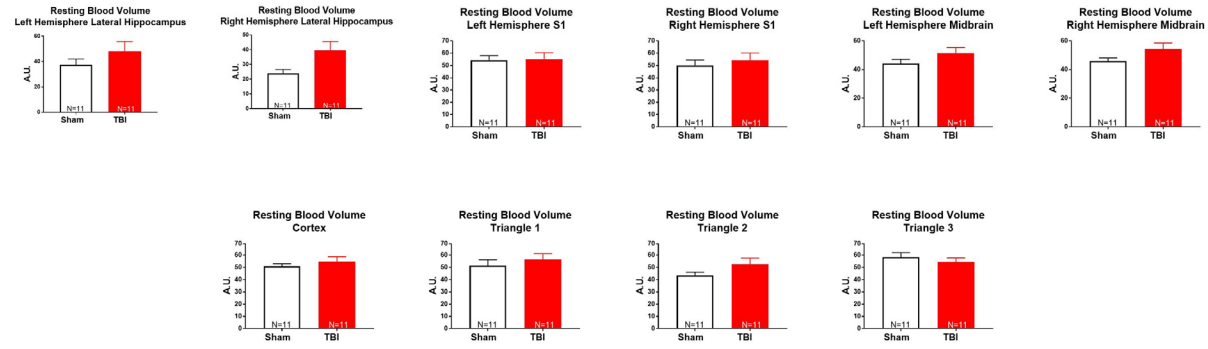

Fig. 3. Resting regional blood volume in selected regions of the brain. All p=NS.

Subtask 4: Conduct ex vivo vasoreactivity of isolated circle of Willis arteries from TBI and uninjured rats.

Accomplishments:

Vasoreactivity data were obtained for all rat cohorts.

Preliminary Scientific Findings: Pial (circle of Willis) arterial myogenic tone was determined by progressive exposure of cannulated arteries to 30 and 60 mm Hg (physiologic pressures). Myogenic tone was not different between sham and TBI groups. Similarly, no difference was found in baseline endothelium-dependent dilation between TBI and sham rats. When including all rats in all cohorts, there was significant impairment in smooth muscle-dependent dilation in TBI rats versus sham rats (51.6±18% versus 42.7±16%, p=0.02) (Fig. 5). Results of dilator response post-exposure to Aβ42 and high glucose are discussed in the following sections.

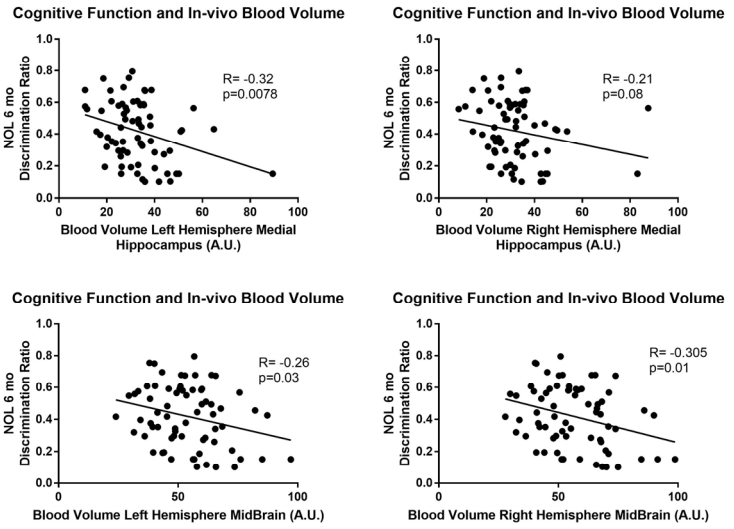

Fig. 4. There was inverse relationship between resting blood volume in medial hippocampus and NOL and midbrain (diencephalon) and NOL, a measure of long term memory.

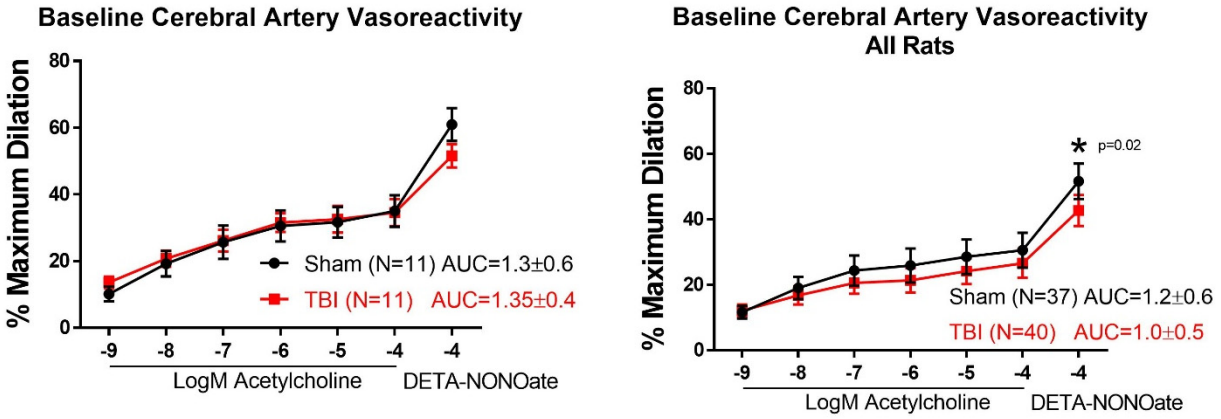

Fig. 5. Ex-vivo baseline vasoreactivity of circle of Willis arteries. There was no significant difference in baseline endothelium-dependent function between TBI and sham rats. When including all rat cohorts tested, smooth muscle-dependent function was modestly impaired in TBI rats.

Subtask 5: Perform neuropathological assessment of brain hemispheres, including laser capture microdissection and initial gene expression assays.

Accomplishments: We collected *brain microvessels* using laser microdissection from TBI and sham rats (N=5) each and performed RNAseq analyses. Top 10 differentially expressed genes are shown in **Table 1**. Note that the false discovery rate values of ~0.1 is an acceptable threshold for initial screening of genes for future validation. We will perform gene set enrichment analyses to determine association pathways. Preliminarily, the results suggest an association with hypertension, subdural hemorrhage and insulin signaling, although confirmation is needed. We plan to confirm the top

candidate genes by qPCR. We are also in the process of laser microdissection of vessels and microglia in selected rats with TBI/sham and STZ/noSTZ for RNA analyses. We anticipate having some of these data by next reporting quarter.

Table 1 Gene	Gene name	Gene Function	logFC	logCPM	LR	PValue	FDR
Gstt3	Glutathione S-transferase theta-3	Conjugation of reduced glutathione	16.974	6.78825427	19.392753	1.064E-05	0.1386714
Aldh3a2	aldehyde dehydrogenase 3 family member A2	fatty oxidation	16.988	6.80245614	19.066047	1.263E-05	0.1386714
Mag	myelin associated glycoprotein	myelination process	17.19	7.00448094	19.049525	1.274E-05	0.1386714
Slc30a3	solute carrier family 30 member 3	zinc accumulation in synaptic vesicles	18.473	8.2877259	18.213496	1.975E-05	0.1612478
Lrfn5	leucine rich repeat fibronectin III domain containing 5	presynaptic differentiation	17.496	7.31073704	17.668265	2.63E-05	0.1639674
Tns3	tensin 3	actin remodeling	16.486	6.30089171	16.932594	3.873E-05	0.1639674
Mtss1	MTSS I-BAR domain containing 1	interaction with the actin cytoskeleton	16.789	6.60412259	16.706678	4.363E-05	0.1639674
Inpp4a	inositol polyphosphate-4-phosphatase type I A	Protein Coding gene	16.635	6.44961188	16.640284	4.518E-05	0.1639674
Fdx1l	ferredoxin 2	heme A and Fe/S protein biosynthesis	17.237	7.05166895	16.154377	5.838E-05	0.1906922

3. Identify potential mechanisms of TBI-induced cerebrovascular dysfunction by assessing vascular oxidative/nitrative stress and vascular inflammation following TBI and assess their relationship to cognitive dysfunction.

Subtask 1: Assess blood samples for markers of oxidative stress and inflammatory markers.

Identified challenges: Even with use of highly sensitive nitrite assay (sensitivity down to 20 picomol level), we found serum levels in rats to be in the lowest range of standards, suggesting very low levels of circulating NO (as determined by nitrite levels) even with our sensitive nitrite assay. We are continuing to optimize this process. We remain realistic that this measurement may not be possible in blood samples 6 month post-injury.

Accomplishments: Blood draws are complete for all rat cohorts. We plan on performing batch assay for inflammatory markers. Due to the small volume of rat sera, we plan on doing multianalyte assay (instead of ELISA) using Luminex method which we anticipate being in our lab by early 2021.

Subtask 2: Evaluate oxidative and nitrative stress in circle of Willis arteries

Accomplishments: Arteries from TBI and sham rats have been processed and results reported in last annual report. Separate circle of Willis arterial segments were isolated, treated with vehicle, Aβ42 or high glucose and exposed to hydroethidine (superoxide marker), dihydrorhodamine (peroxynitrite marker) and DAF-2 (nitric oxide marker) for immunofluorescence imaging. Results for TBI versus sham showed no significant difference in baseline (vehicle-treated) cerebral artery superoxide, peroxynitrite and NO between TBI and uninjured rats. There was also no difference following exposure to high glucose. However, following exposure to Aβ42, there was significant increased production of peroxynitrite and trend towards increased superoxide in TBI vessels, suggesting increased predisposition to nitrative stress in TBI when exposed to Aβ42. We have collected the arteries from TBI/sham rats treated with STZ or vehicle and imaging analyses of these dataset are pending.

Subtask 3: Quantify inflammation through gene and protein expression analyses of inflammatory markers in circle of Willis arteries.

Accomplishments: Samples have been collected from TBI and sham and will be completed in LPS pretreated rats. Tissue preparations for IHC analyses are ongoing.

Subtask 4: Measure smooth muscle contractile proteins and eNOS gene and protein expression in TBI and sham groups.

Accomplishments: Following collection of circle of Willis arteries from cohorts 1-2, we now recognize that we will have insufficient arterial mass after vasoreactivity and oxidative stress assays to perform PCR gene expression assays. It is not possible to perform gene expression assays on these arteries. The same limitation holds for Western blot assay. We did collect arterial segments fixed in paraformaldehyde for immunohistochemistry that will allow protein assays by IHC or immunofluorescence.

Results so far show no difference in circle of Willis pial arterial smoothelin (smooth muscle contractile protein) content (sham versus TBI: 21.5±6.1% vs. 21.3±7.1% arterial wall area, p=NS, N=14 each). Incomplete results for phospho-NFKB showed no significant difference but with a trend towards higher values in TBI versus sham (1.76±0.5 vs. 1.13±0.4 A.U. by visual scoring, p=NS, N=6 and N=9, respectively). Quantification of IL-8 content remains ongoing, as well as quantification of the rest of the rat cohorts.

4. Evaluate whether preconditioning with lipopolysaccharide will attenuate TBI-induced cerebrovascular dysfunction and inflammation and prevent TBI-mediated cognitive dysfunction.

Subtask 1: Produce cohorts of uninjured and TBI LPS preconditioned rats with blood collection and cognitive function assessments.

Subtask 2: Conduct in vivo MRI vascular function, ex vivo vasoreactivity and neuropathology.

Accomplishments: We completed this cohort. Contrary to our hypothesis that LPS pretreatment would prime protective "hormesis"-type response in TBI, LPS pretreatment led to worse cognitive

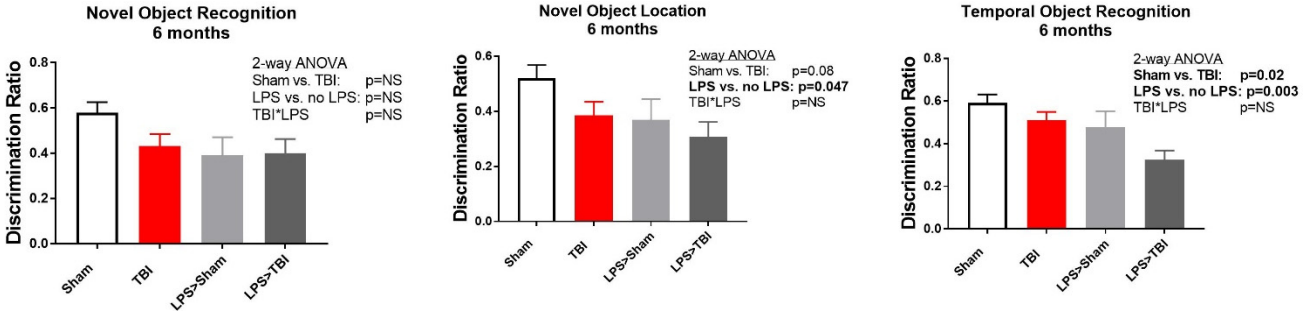

Fig. 6. Cognitive function tests show that pre-treatment with LPS did not result in improvement of cognitive function in TBI rats. Contrary to hypothesis, LPS pretreatment resulted in worse cognitive function (NOL and TOR).

function (Fig. 6). Potential translational significance is possible predisposition to worse long term cognitive dysfunction in soldiers or veterans with baseline systemic inflammatory state (e.g. obesity, vascular inflammation/atherosclerosis, periodontitis) who sustains a TBI. This effect should therefore be explored further.

5. Compare the ex-vivo responses of cerebral arterioles between uninjured and TBI rats following exposure to high glucose and Aβ42.

Subtasks 1-2: Test vascular function of cerebral vessels of TBI vs. uninjured when exposed to high glucose or Aβ.

Subtask 3: Assess oxidative and nitrative stress and inflammation following exposure to HG or Aβ.

Accomplishments: There was a trend, but no significant difference, towards worse dilator response to maximum acetylcholine dose following exposure to Aβ42 in TBI versus sham rats, with no

significant difference in response to NO donor DETA-NONOate (Fig.7). There was no difference between TBI and uninjured responses following exposure to high glucose (Fig. 7).

6. Compare cerebrovascular function, vascular inflammation and cognitive function in stretptozotocin-treated rats (diabetes model) which had antecedent TBI versus no injury.
Subtasks 1-6: produce cohorts of uninjured and TBI rats, measure cognitive function prior to 90 days, inject stretptozotocin at 90 days, measure cognitive function at 180 days, followed by in vivo and ex-vivo vascular function and neuropathological assessment.
Accomplishments: We have completed measuring cognitive function, in vivo and ex vivo cerebrovascular function in these cohorts. As we anticipated, STZ

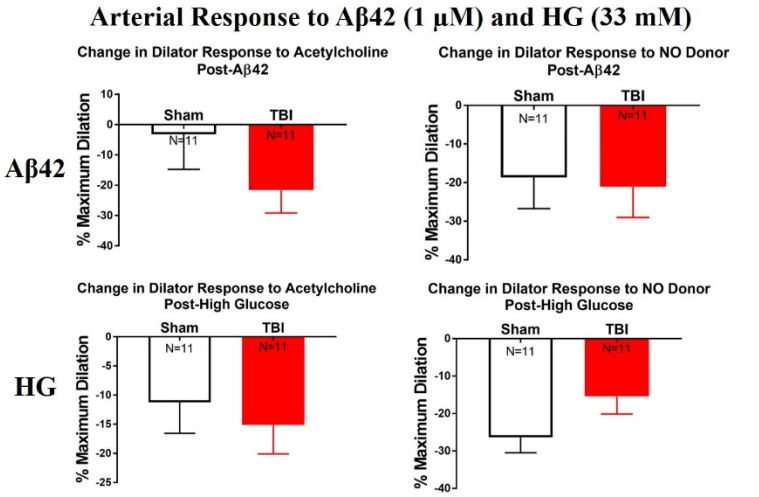

Fig. 7. CoW arterial response following exposure to Aβ42 and high glucose. There was a trend, but not significant, of worse dilator response to max. acetylcholine in TBI versus sham. There was no significant difference in response to high glucose between sham and TBI.

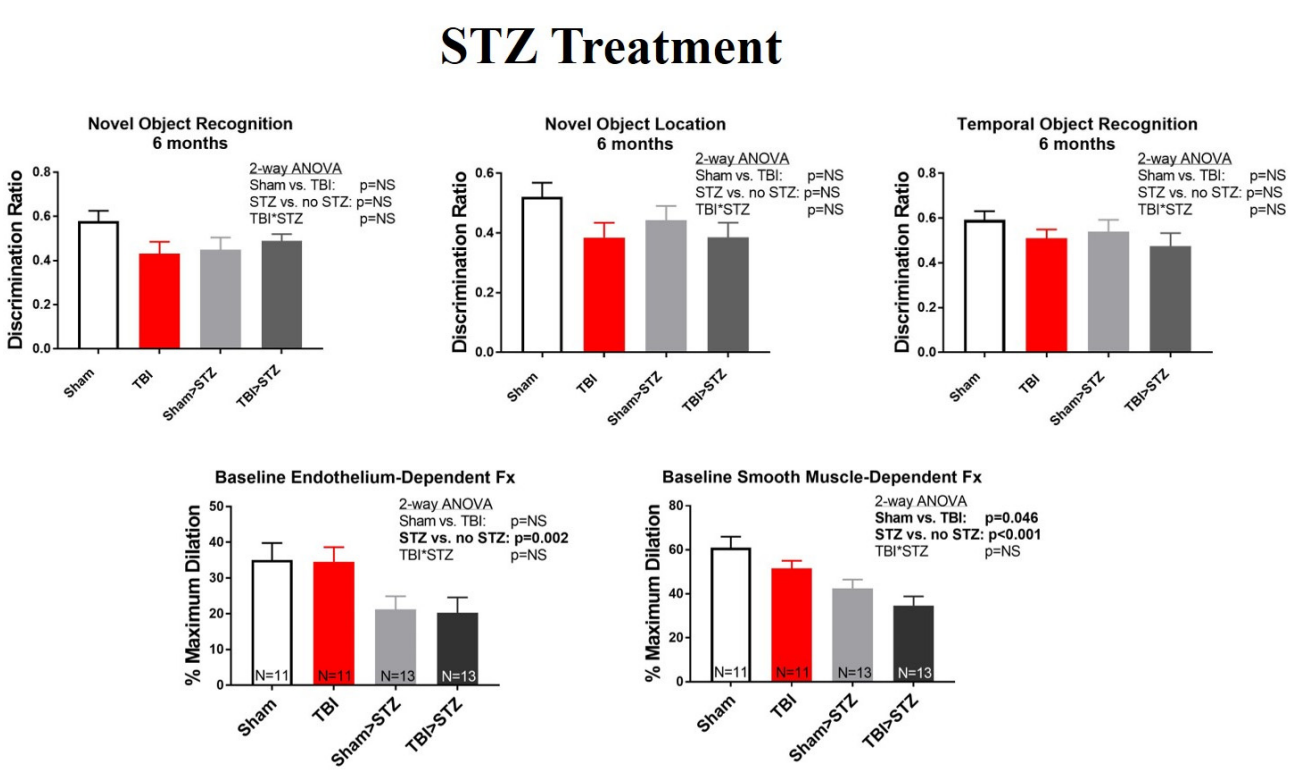

Fig. 8. Upper panel shows cognitive function assays in rats treated with TBI, sham, TBI followed by STZ and sham followed by STZ. There was no significant difference in NOR, NOL and TOR in STZ versus no STZ treated rats. Lower panel shows ex-vivo baseline vasoreactivity showing significant difference in endothelial and smooth muscle dependent function in STZ versus no-STZ rats, and significant difference in smooth muscle dependent function in TBI versus sham rats. The effects of TBI x STZ on smooth muscle function appears additive, not synergistic.

treatment and subsequent hyperglycemia development was very stressful to the rats and as we anticipated in the study design, there were more non-completers in this cohort versus the other cohorts that did not involve STZ administration. Although there was a trend towards worse NOL and TOR scores in STZ treated rats (Fig 8, upper panel), 2 way ANOVA showed no significant difference in NOR, NOL and TOR by STZ treatment. We did not see any significant interaction between STZ treatment and TBI exposure, contrary to our hypothesis. For baseline cerebrovascular function, we found significant worsening of baseline endothelium-dependent and smooth muscle-dependent function in STZ-treated rats, and worse smooth muscle dependent function in TBI versus sham rats (Fig. 8, lower panel). Since the interaction term for TBI x STZ exposure was not significant, we interpret our data as TBI and diabetes induction (following STZ) cause additive, but not synergistic, worsening of cerebrovascular (pial artery) smooth muscle function.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The Translational Neurotrauma Research Program hosts a **monthly community journal club** to discuss neurotrauma from all aspects. We have attendees who represent the legal profession, physical therapy, drug companies, physicians, scientists, and trainees. Over the last academic year, the topics included: employment after TBI, addiction and TBI, psychosocial outcomes in pediatrics and TBI, environment and weather, tumorigenesis, biomarkers, the vegetative state, maternity, and transmissible disease.

Lifshitz Lab: Conor Young is the primary technician on this project. He joined the group in February of 2018, and has coordinated all the physiology, behavior, transport, and tissue dissections for each cohort. He has become the primary point person for animal status on the project. He has completed his tenure with the group and will continue to contribute to the publication of the data.

Migrino Lab: Michael Hansen, Research Technician, has acquired technical training and new skillsets in lab methods such as Western blot, tissue preparation, immunohistochemistry and microscopy, broadening his professional experience. He has left the team to pursue advanced studies.

Quarles Lab: Alberto Fuentes is a Masters student at Arizona State University who is analyzing the MRI data for this project. He has gained expertise in rat brain image co-registration, atlas-based region of interest analysis, contrast agent-based perfusion modeling and cerebral vascular reactivity analysis.

Mastroeini Lab: Jennifer Nolz and Elaine Delvaux have acquired new skillsets in processing rat tissues specifically in identification and isolation of cerebral vessels using laser microdissection as well identifying specific gene transcripts appropriate for the study design and aims.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Dr. Migrino presented preliminary results to the DOD Meeting in Ft Detrick, MD in August 2019. An abstract and poster of our results was presented in the 2020 15th Annual NABIS Conference on Brain Injury. This conference was the last available prior to the COVID-19 cancellation of most in-person conferences.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will complete brain tissue and cerebral arterial preparation and processing and do batch assays of blood and tissue analyses. Data will be organized with the oversight of our statistician (Dr. Hu) to begin modeling the main effects of injury.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The major impact of our project is the validation and characterization of this model of TBI, previously considered to be "mild" type due to lack of overt post-acute neuropathologic changes from previous studies, as actually leading to chronic cognitive and vascular changes that would merit its re-classification as a "mild" injury. Ongoing discussions define the midline fluid percussion model as a complicated mild TBI with persistent post-concussive symptoms. Our results validate the importance of this model by recapitulating observations in human subjects, and importantly, by showing the coexistence and associational relationship of chronic cognitive function and vascular function changes following TBI. We still have to complete our work to tease out potential mechanistic relationship between vascular and cognitive dysfunction in this model. We also show that TBI and

our diabetes model (STZ treatment) cause additive impairment in cerebrovascular smooth muscle function which may have translational relevance in soldiers and veteran who sustain TBI at a younger age and who are predisposed to later development of diabetes.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Currently existing scientific silos delineated by disparate disciplines (neuroscience versus cardiovascular disease) limit discoveries on causal mechanisms within each specific discipline. The findings from our multidisciplinary group hopefully will enhance understanding of the shared mechanisms at the intersection of these 2 disciplines to further our knowledge of the pathophysiology of TBI.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

1. As stated previously, following collection of circle of Willis arteries from cohorts 1-2, we now recognize that we will have insufficient arterial mass after vasoreactivity and oxidative stress assays to perform PCR gene expression assays so we will not be able to perform gene expression assays on these arteries. The same limitation holds for Western blot assay. We did collect arterial segments fixed in paraformaldehyde for immunohistochemistry that will allow us to protein assays by IHC or immunofluorescence. We will also do gene expression assays on isolated parenchymal cerebral vessels.
2. We have adopted a clinically-relevant 6 hour fasting procedure prior to blood collection necessary to monitor the diabetes arm of the study.
3. Two stages of hypercapnic blood flow studies will be conducted in order to identify relationships between ~1% and 5% CO_2, which are necessary to determine the trajectory of cerebrovascular responses to TBI. Following discovery of our faulty hypercapnea protocol in the initial cohorts, we corrected the process for the following cohorts.
4. We knew of no precedent on how to deal with scoring cognitive function discrimination for rats who have no interest at all in exploring objects. This response is clearly a very abnormal response as physiologically normal rats explore their environment in order to survive. Not exploring objects will not provide a discrimination score. We dealt with this issue by recognizing that such a response is very abnormal and imputing a score that reflects this condition. For data imputation, we utilized the lowest time for object exploration across all animals in a group divided by the longest time for object exploration across all animals in a group. This will result in the smallest discrimination ratio possible for that group and time point. For manuscripts, we will provide analyses based on this analysis and provide separate analysis for data without imputation.
5. A cohort of rats were not imaged as scheduled at 6 months but had to be postponed to 9 months post injury due to extremely rare MRI quenching episode at Barrow Neurological Institute. For our analyses, we excluded imaging and ex-vivo data on these 9 months rat to ensure comparability of groups at the 6 month timeframe.
6. The COVID19 epidemic created work restrictions in all facilities. Fortunately for our group, the very valuable (and hard to replace) existing cohort of rats treated with TBI and STZ prior to COVID19 were allowed to be processed for data acquisition in all facilities, and we were able to proceed with critical measurements. We did have to delay processes that could be postponed without scientific detriment, such as processing of frozen or formaldehyde preserved tissue, image analyses, etc.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Because of the preliminary and incomplete nature of the dataset, no publication has directly resulted from this project proposal.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Abstract presented "Experimental TBI induces long term cognitive deficits and vascular pathology" at the 2020 15th Annual NABIS Conference on Brain Injury. Manuscripts are in preparation stage.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

In light of DOD grant support which provided Dr. Migrino protected research time and allowed continued employment of research associates (Seth Truran and Nina Karamanova), the DOD grant indirectly supported the publication of the following work, which we acknowledged in the manuscripts:

1. Migrino RQ, **Karamanova N**, **Truran S**, Serrano GE, Davies HA, Madine J, Beach TG. Cerebrovascular medin is associated with Alzheimer's disease and vascular dementia. Alzheimer's Dement. 2020; 12:e12072. .

2. **Karamanova N, Truran S**, Serrano GE, Beach TG, Madine J, Weissig V, Davies HA, Veldhuizen J,Nikkhah M, Hansen M, Zhang W, D'Souza K, Franco DA and **Migrino RQ**. Endothelial Immune Activation by Medin: Potential Role in Cerebrovascular Disease and Reversal by Monosialoganglioside-Containing Nanoliposomes. J Am Heart Assoc. 2020;9:e014810.

3. Younger S, Jang H, Davies HA, Niemiec MJ, Garcia JGN, Nussinov R, **Migrino RQ**, Madine J, Arce FT. Medin oligomer membrane pore formation: a potential mechanism of vascular dysfunction. Biophysics J. 2020; 118:2769-2782.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Seth Truran
Project Role:	Research Associate
Nearest person month worked:	3.0x4
Contribution to Project:	Mr. Truran worked on logistical coordination, methodologic optimization of vascular procedure and tissue collection process required for the project.

Name:	Nina Karamanova DVM
Project Role:	Research Associate
Nearest person month worked:	1.05x4
Contribution to Project:	Dr. Karamanova worked with Mr. Truran on logistical coordination, methodologic optimization of vascular procedure and tissue collection process required for the project.

Name: Karen D'Souza, PhD

Project Role: Research Technician
Nearest person month worked: 0.45x4
Contribution to Project: Dr. D'Souza worked with Mr. Truran on logistical coordination, methodologic optimization of vascular procedure and tissue collection process required for the project.

Name: Gail Farrell
Project Role: Research Coordinator-Backup
Nearest person month worked: 0.08x4
Contribution to Project: Ms. Farrell assisted as needed with local regulatory requirements for the project.

Name: Peter Reaven
Project Role: Co-investigator
Nearest person month worked: 0.15x4
Contribution to Project: Dr. Reaven helped optimize protocols and methodology as well as data interpretation.

Name: L Matthew Law, PhD
Project Role: Post-doctoral fellow
Nearest person month worked: 0.6x4
Contribution to Project: Dr. Law is responsible for cohort planning, animal behavioral testing, and overall management of animal work. He is the primary communication for Dr. Lifshitz.

Name: Daniel Griffiths
Project Role: Research Technician
Nearest person month worked: 0.3x4
Contribution to Project: Mr. Griffiths performs the animal surgery and injury and is responsible for the day-to-day supply orders to conduct studies.

Name: Conor Young
Project Role: Research Technician
Nearest person month worked: 2.55x4
Contribution to Project: Mr. Young is a new technician assisting Dr. Law and Mr. Griffiths, while training on all animal procedures.

Name: Raymond Migrino MD
Project Role: Joint PI
Nearest person month worked: 0.6x4
Contribution to Project: As joint and corresponding PI, direction, supervision and logistical administration of the project with multiple partner scientists and institutions.

Name: Jonathan Lifshitz PhD
Project Role: Joint PI

Nearest person month worked: 0.3x4
Contribution to project: Supervision and organization of initiation of first animal cohorts including personnel supervision and administrative/regulatory functions. As joint PI, logistical administration of the project with multiple partners.

Name: Rayna Gonzales
Project Role: Co-investigator
Nearest person month worked: 0.15x4
Contribution to Project: Dr. Gonzales helped optimize protocols and methodology as well as data interpretation.

Name: Chengcheng Hu
Project Role: Co-investigator
Nearest person month worked: 0.15x4
Contribution to Project: Dr. Hu helped provide data and statistical input and support.

Name: C. Chad Quarles, PhD
Project Role: Co-investigator
Nearest person month worked: 0.6x4
Contribution to project: Worked on imaging protocol optimization and validation in preparation for the first cohort of animals to be transferred to Barrow.

Name: Gregory Turner PhD
Project Role: Co-investigator
Nearest person month worked: 0.3x4
Contribution to project: Dr. Turner directed and performed brain imaging protocols including optimization and problem solving of neuroimaging procedures.

Name: Alberto Fuentes
Project Role: Research Engineer II
Nearest person month worked 1.0x4
Contribution to project: Worked with Dr. Quarles on quantification of brain imaging data including optimization of measurement protocols

Name: Diego Mastroeni, PhD
Project Role: Co-investigator
Nearest person month worked: 0.6x4
Contribution to project: Developed and optimized protocols for Immuno-laser capture Microdissection on vascular cells.

Name: Jennifer Nolz
Project Role: Research Technician
Nearest person month worked: 1.5x4
Contribution to project: Methodologic optimization of vascular laser capture microdissection procedure and tissue sectioning and processing.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No changes for Migrino, Lifshitz, Reaven, Gonzales, Turner.
Changes for Quarles not included in first annual report:
Dignity Health and ASU Collaborative Strategic Initiatives: PI: Quarles, 9/24/18-9/23/19 (1.2 calendar months)

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

The following 4 organization are partners in the current project, each identified by key personnel and funds awarded to each institution.
Organization Name: Phoenix VA Healthcare System
Location of Organization: Phoenix, AZ
Partner's contribution to the project (identify one or more): Collaboration

Organization Name: University of Arizona College of Medicine - Phoenix
Location of Organization: Phoenix, AZ
Partner's contribution to the project (identify one or more): Collaboration

Organization Name: Barrow Neurological Institute
Location of Organization: Phoenix, AZ
Partner's contribution to the project (identify one or more): Collaboration

Organization Name: Arizona State University
Location of Organization: Tempe, AZ
Partner's contribution to the project (identify one or more): Collaboration

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

STATEMENT OF WORK AND % COMPLETION OF WORK

Site 1: **Phoenix VA Healthcare System [PVAHCS]**
650 Indian School Rd
Phoenix, AZ 85012
PI: Ray Migrino, MD

Site 2: **University of Arizona, College of Medicine – Phoenix [UA COM-P]**
425 N. 5th St.
Phoenix, AZ 85004
PI: Jonathan Lifshitz, PhD

Site 3: **Arizona State University [ASU]**
727 E. Tyler Street
Tempe, AZ 85287-5001
PI: Diego Mastroeni, PhD

Site 4: **Barrow Neurological Institute [BNI]**
350 W. Thomas Rd.
Phoenix, AZ 85013
Christopher Quarles, PhD

Research-Specific Tasks:	**Months**	**% Completion**
Major Task 1: Obtain institutional and DOD approval for live animal work	1-3	
Milestone(s) Achieved: Obtain IACUC approval and DOD ACURO approval	3	100%
Specific Aim 1: In rats exposed to midline fluid percussion injury (FPI), evaluate the extent and mechanisms of cerebrovascular dysfunction and inflammation and establish their relationship with cognitive function.		
Major Task 2: Compare 180-day in-vivo cerebral flow (CBF) and cerebrovascular reactivity using MRI, and ex-vivo endothelial and smooth muscle-dependent function of isolated circle of Willis cerebral arteries from TBI versus uninjured rats and determine the relationship of vascular function with measures of cognitive function (novel object recognition tasks) and degree of neuropathology. **Aim 1: Number of experimental groups: 3 (Group 1 TBI, Group 2 Sham control, Group 3 LPS>TBI group)** **Number of rats per group: 12 (total 36 with complete data)***		
Subtask 1: Produce cohorts (n=12) of uninjured and diffuse brain-injured rats using midline fluid percussion with inclusion criteria of acute neurological reflex suppression and transient motor impairments.	3-9	100%
Subtask 2: Draw submandibular blood monthly and conduct behavioral battery of cognitive testing (novel object recognition) at 3 months and 6 months post-injury.	4-12	100%
Subtask 3: Conduct in-vivo cerebral flow (CBF) and cerebrovascular reactivity using MRI between brain-injured and uninjured rats.	9-15	100%
Subtask 4: Conduct ex-vivo endothelial and smooth muscle-dependent function of isolated circle of Willis cerebral arteries from TBI versus uninjured rats.	9-15	100%
Subtask 5: Perform neuropathological assessment of brain hemispheres, including laser capture microdissection and initial gene expression assays.	9-18	80%
Milestone(s) Achieved: Defined relationship between vascular function (in vivo and ex vivo) and cognitive function, supported by neuropathology; publication of 1 peer reviewed paper.	18	95%
Major Task 3: Identify potential mechanisms of TBI-induced cerebrovascular dysfunction by assessing vascular oxidative/nitrative stress and vascular inflammation following TBI and assess their relationship to development of cognitive dysfunction		

Subtask 1: Assess blood samples for systemic markers of oxidative stress (malondialdehyde; superoxide dismutase; glutathione peroxidase) and inflammatory markers (IL-1B; IL-6; IL-8; C-reactive protein) by ELISA	10-16	20%
Subtask 2: Evaluate oxidative and nitrative stress in isolated circle of Willis cerebral arteries using immunofluorescence microscopy for NO, superoxide and peroxynitrite (using hydroethidium, diaminofluorescein-2 and coumarin boronate fluorescence, respectively)	10-16	80%
Subtask 3: Quantify inflammation using gene expression of RAGE, IL-1B, IL-6, IL-8 and protein expression of phosphorylated NFκB and RAGE in Circle of Willis arteries	10-16	90%
Subtask 4: Measure smooth muscle contractile proteins (MHC and smoothelin) and endothelial cell proteins relevant to vasoreactivity (total and phosphorylated endothelial and inducible nitric oxide synthases, eNOS and iNOS) by gene and protein expression between TBI and sham groups.	10-16	75%
Milestone(s) Achieved: Defined relationship between potential mechanisms of TBI-induced cerebrovascular dysfunction the development of cognitive dysfunction; publication of 1 peer reviewed paper.	18	80%
Major Task 4: Evaluate whether preconditioning with lipopolysaccharide (LPS) (a well-established and validated method to reduce brain injury through vascular protection and enhanced NO bioavailability) will attenuate TBI-induced cerebrovascular dysfunction and inflammation and prevent TBI-mediated cognitive dysfunction		
Subtask 1: Produce cohorts of uninjured and diffuse brain-injured and LPS-preconditioned rats (0.5 mg/kg i.p.) with blood collection and cognitive function assessments	18-27	100%
Subtask 2: Conduct in vivo vascular function, ex vivo vasoreactivity, and neuropathology.	24-27	100%
Milestone(s) Achieved: Identification of role for inflammatory pre-conditioning in preserving vascular function after TBI	18	100%
Specific Aim 2: To determine whether TBI and diabetes-related metabolic derangements or β-amyloid confer synergistic deleterious effects on cerebrovascular function, inflammation and cognitive function.		
Major Task 5: Compare the responses of ex-vivo circle of Willis arteries from uninjured and brain-injured rats without and with acute exposure to CVRF (high glucose) and β-amyloid (Aβ42) in terms of endothelial and smooth-muscle function, oxidative and nitrative stress and pro-inflammatory signaling.		
Subtask 1: Test vascular function of ex vivo cerebral vessels from TBI and uninjured rats when exposed to 1 hour of high glucose	10-16	100%
Subtask 2: Test vascular function of ex vivo cerebral vessels from TBI and uninjured rats when exposed to Aβ (Aβ40 or Aβ42) at two doses.	10-16	100%
Subtask 3: Expose ex vivo vessels to high glucose or Aβ for 1 or 24 hours and measure oxidative and nitrative stress (SO, NO, ONOO, eNOS) and inflammation (IL-6, IL-8, IL1B, NFκB, RAGE) by gene and/or protein expression.	11-17	90%
Milestone(s) Achieved: Determine whether ex vivo cerebral vessels isolated from injured rats have worse endothelial function when exposed to high	20	90%

glucose or Aβ as compared to uninjured rats; publication of 1 peer reviewed paper		
Major Task 6: Compare cerebrovascular function, vascular inflammation and cognitive function in rats with Streptozotocin-induced type 2 diabetes which had antecedent TBI versus uninjured rats **Aim 2: Number of experimental groups: 2 (Group 4 TBI>DM, Group 5 Sham>DM)** **Number of rats per group: 24, (total of 48 with complete data)***		
Subtask 1: Produce cohorts (n=24 each) of uninjured and diffuse brain-injured rats using midline fluid percussion with inclusion criteria of acute neurological reflex suppression and transient motor impairments.	21-27	100%
Subtask 2: Inject Streptozotocin (65 mg/kg, i.p.) at 90 days post-injury to induce type 2 diabetes mellitus.	22-30	100%
Subtask 3: Draw submandibular blood monthly and conduct behavioral battery of cognitive testing (novel object recognition) at 3 months and 6 months post-injury.	22-30	100%
Subtask 4: Conduct in-vivo cerebral flow (CBF) and cerebrovascular reactivity using MRI between brain-injured and uninjured rats.	27-33	100%
Subtask 5: Conduct ex-vivo endothelial and smooth muscle-dependent function of isolated circle of Willis cerebral vessels from TBI versus uninjured rats.	27-33	100%
Subtask 6: Perform neuropathological assessment of brain hemispheres, including laser capture microdissection and initial gene expression assays.	27-33	30%
Milestone(s) Achieved: Determined whether diabetic rats with preceding TBI have worse cognitive function and cerebrovascular function when compared to diabetic rats without preceding TBI or injured (TBI) rats; publication of 1 peer reviewed paper.	36	80%

Probing the Mechanistic Role of Vascular Dysfunction and Vascular Inflammation in TBI-Mediated Cognitive Dysfunction

W81XWH-17-1-0473

PI: Raymond Migrino/Jonathan Lifshitz **Org:** Carl T. Hayden Medical Research Foundation **Award Amount:** $1,300,000

Study Aims

Aim 1: In rats exposed to midline fluid percussion injury (FPI), evaluate the extent and mechanisms of cerebrovascular dysfunction and inflammation and establish their relationship with cognitive function.

Aim 2: To determine whether TBI and diabetes-related metabolic derangements or β-amyloid confer synergistic deleterious effects on cerebrovascular function, inflammation and cognitive function.

Approach

1A. Compare 6-month cerebral flow, and ***ex-vivo*** function of cerebral arteries from TBI versus uninjured rats and determine the relationship between vascular function with cognitive function.

1B. Identify mechanisms of TBI-induced cerebrovascular dysfunction by assessing oxidative and inflammation following TBI.

1C. Evaluate whether preconditioning with lipopolysaccharide will attenuate TBI-induced cerebrovascular dysfunction and inflammation and prevent TBI-mediated cognitive dysfunction.

2A. Compare the responses of cerebral arteries from uninjured and TBI rats without and with acute exposure to high glucose or β-amyloid.

2B. Compare cerebrovascular function, vascular inflammation and cognitive function in rats with streptozotocin-induced type 2 diabetes which had antecedent TBI versus uninjured rats.

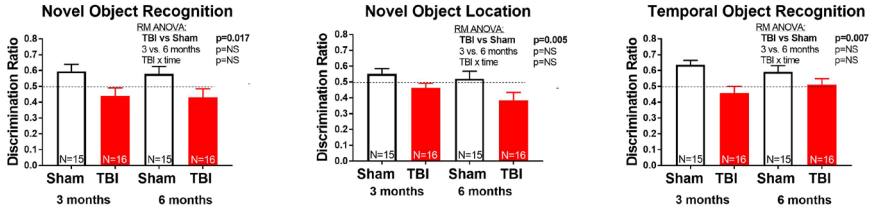

Fig. 1. Cognitive function tests show impaired NOR, NOL and TOR in TBI versus sham at 3 and 6 months.

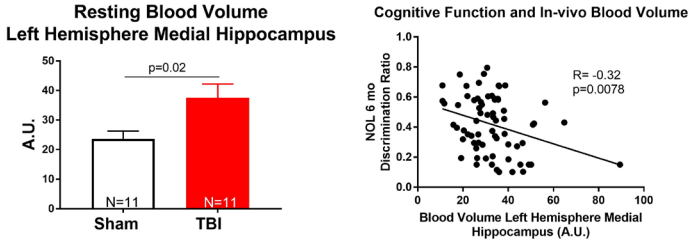

Fig. 2. In-vivo MRI showed increased blood volume in hippocampus in TBI rats. NOL, a measure of long-term memory, is inversely related to resting hippocampal blood volume in this cohort.

Timeline and Cost

Activities CY	17	18	19	20
Compare vascular function in TBI vs Sham				
Identify mechanisms of TBI vascular dysfunction				
Assess role of LPS in TBI pathophysiology				
Assess modulating role of metabolic risk factors in TBI and cognitive dysfunction				
Estimated Budget ($K)	**$50**	**$420**	**$420**	**$410**

Updated: 08/26/2020

Goals/Milestones

CY17 Goal – Project Initiation
- ☑ Obtain institutional and DOD ACURO approval
- ☑ Initiate first cohort of uninjured and TBI injured rats

CY18 Goals – Assess vascular function and cognition in TBI
- ✓ Compare CBF and vascular function in TBI vs. sham
- ✓ Probe mechanisms of vascular dysfunction in TBI

CY19 Goal – Assess modulating roles of LPS and HG in TBI
- ✓ Probe effects of LPS and streptozotozin in TBI vascular and cognitive dysfunction

CY20 Goal – Establish mechanistic link between vascular and cognitive dysfunction in TBI
- ☐ Determine relationship and mechanisms of linkages

Comments/Challenges/Issues/Concerns
- COVID19-related on-site work lab restrictions

Budget Expenditure to Date; July 31, 2020

Projected Expenditure: $1,300,000

Actual Expenditure: $896,008.37