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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 all 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. Altered pial arterial smooth muscle function post-angiotensin II was seen 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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1. **INTRODUCTION:**

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:**

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

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Please see attachment.

What was accomplished under these goals?

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. Data have been analyzed and compared between groups, including correlation analysis. Results have been written and published in J of Neurotrauma (PMID 35593008).
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 NOR at 3 months that persist up to 6 months, with similar trend (not statistically significant) for NOL and TOR (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.

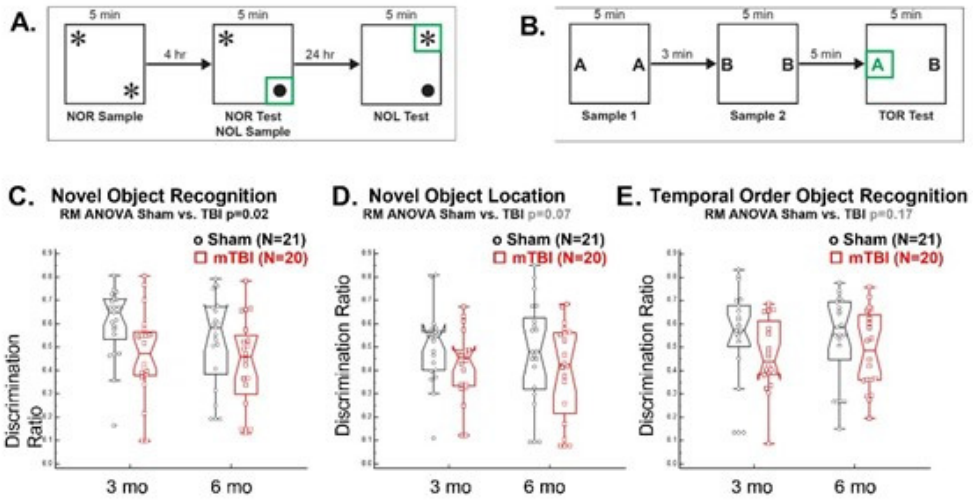


Fig. 1. Chronic cognitive impairment following mTBI. A. Schematic of object recognition tasks. Novel object recognition (NOR) tests short term memory by replacing an object (*) with (●) after a 4-hour delay. Novel object location (NOL) tests long term memory by shifting the position of the familiar object (*) after a 24-hour delay. B. Temporal order object recognition (TOR) tests working memory by presenting pairs of objects. C. There was impaired novel object recognition at 3 and 6 months in rats subjected to mTBI versus sham controls. D-E. There were similar trends but not statistically significant differences in novel object location and temporal order object recognition

When all rat cohorts are included (**N=89**) (sham, TBI, sham or TBI without or with LPS, sham or TBI without or with STZ), repeated measures ANOVA showed significantly worse results for TBI treatment for NOR (p=0.003), NOL (p=0.016) and TOR (p=0.026). Our results confirm that mTBI results in persistent chronic cognitive deficits.

Subtask 3: Conduct in vivo cerebral blood flow and cerebrovascular vasoreactivity using MRI in brain injured and uninjured rats.

Accomplishments: We have completed in vivo imaging of all rat cohorts. Results in Figure 2 show no significant difference in global and regional cerebral blood flow between TBI and sham rats, with similar results for regional cerebral blood volume. However, there was significant correlation between cognitive function and regional CBF, where the 6 month NOR significantly correlated with CBF in lateral and medial hippocampus and S1BF. NOL correlated with medial hippocampus NOL (Figure 3). Our results are consistent with previous observations in human concussion patients that CBF is altered in the acute phase of mild TBI but normalizes within 30 days. Our study is not able to answer whether the direct association between cognitive function and regional CBF is causal in nature, which needs further investigation. Persistent abnormality in cognitive function but no significant difference in CBF and CBV at 6 months suggest that the effects of vascular dysfunction are likely relevant in the early stage of the injury.

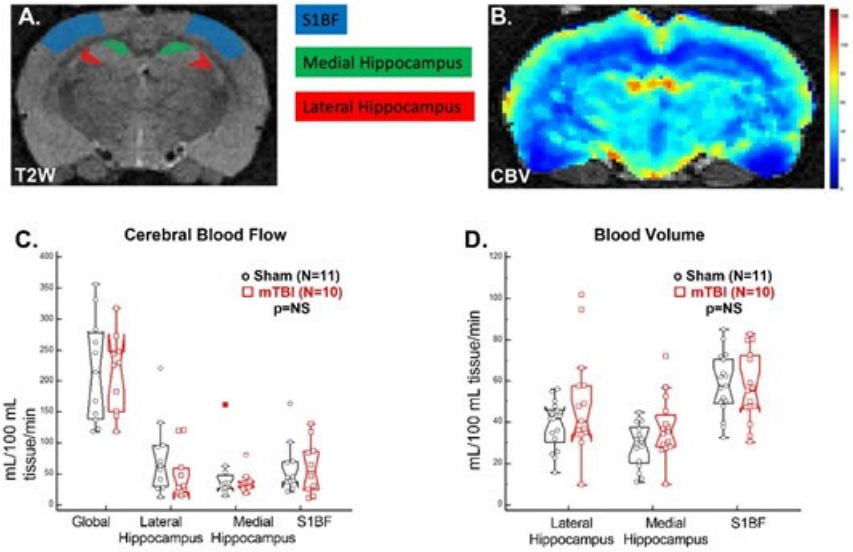


Figure 2. In vivo resting cerebral perfusion. A. Representative T2-weighted anatomic image. The colored areas represent regions of measurement. B. Parametric regional cerebral blood volume map. C. Global and regional resting cerebral blood flow did not differ between mTBI and sham rats at 6 months. D. Regional resting blood volume also did not differ between mTBI and sham rats.

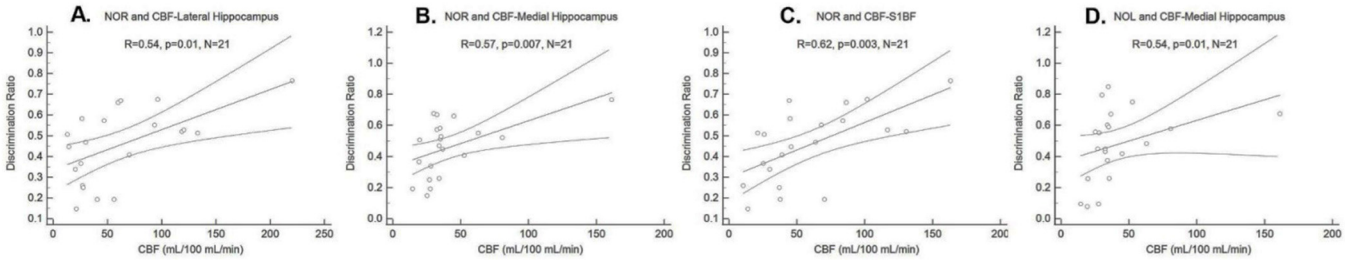


Fig. 3. There was significant correlation between 6 month NOR discrimination score and regional CBF in lateral hippocampus, medial hippocampus and S1BF; there was significant correlation between 6 month NOL and medial hippocampus CBF.

Of interest, the relationship between NOR to regional cerebral blood flow in hippocampal regions is greater in mTBI than sham rats (see table below).

Phenotypic characterization of mild TBI animal model: We previously reported that there is currently no consensus classification schema for TBI severity. Our midline FPI model now adds structural and

functional characterization of a TBI model that we believe would be consistent with current mild TBI

A. Cognitive function and cerebral blood flow

	Sham		mTBI		All	
	R	p-value	R	p-value	R	p-value
NOR-CBF global	0.28	0.28	0.13	0.65	0.17	0.35
NOR-CBF medial hippocampus	0.60	0.05	0.61	0.06	**0.57**	**0.007**
NOR-CBF lateral hippocampus	0.52	0.10	**0.72**	**0.02**	**0.54**	**0.01**
NOR-CBF S1BF	0.51	0.11	**0.87**	**0.001**	**0.62**	**0.003**
NOL-CBF global	0.28	0.28	-0.12	0.66	0.11	0.55
NOL-CBF medial hippocampus	**0.65**	**0.03**	0.36	0.31	**0.54**	**0.01**
NOL-CBF lateral hippocampus	0.40	0.23	-0.33	0.35	0.23	0.32
NOL-CBF S1BF	0.33	0.32	0.13	0.73	0.23	0.31
TOR-CBF global	0.09	0.73	-0.19	0.50	0.01	0.94
TOR-CBF medial hippocampus	0.22	0.51	0.09	0.80	0.18	0.44
TOR-CBF lateral hippocampus	0.45	0.16	-0.07	0.85	0.24	0.30
TOR-CBF S1BF	-0.02	0.95	0.08	0.83	0.12	0.62

classification by VA and DOD standards. Our model relates to non-catastrophic TBI with acute physiologic disruption that recovers within a few days with absence of gross histopathologic damage and lack of cavitation months post injury. In this cohort we found that brain injured animals had righting reflex recovery times of 522±34 (range 245-755 seconds) with sham animals recovering within 15 seconds, injured animals having apnea of 21±3.7 seconds). 6-month brain MRI T2* weighted images showed no evidence of gross bleeding and/or microhemorrhage, while structural images showed no evidence of ventriculomegaly of the third ventricle. Post-mortem brain exam showed lack of gross morphologic features of injury by H&E staining with no evidence of blood extravasation in subcortical white matter. These additional imaging and structural findings 6 months post injury support the mild TBI nature of our animal model and further validates this model for study of mild TBI, a less commonly studied form of TBI.

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. Results show no significant difference in baseline resting pial arterial myogenic tone, endothelium-dependent dilation and smooth muscle-dependent dilation between mTBI and sham rats (Figure 4).

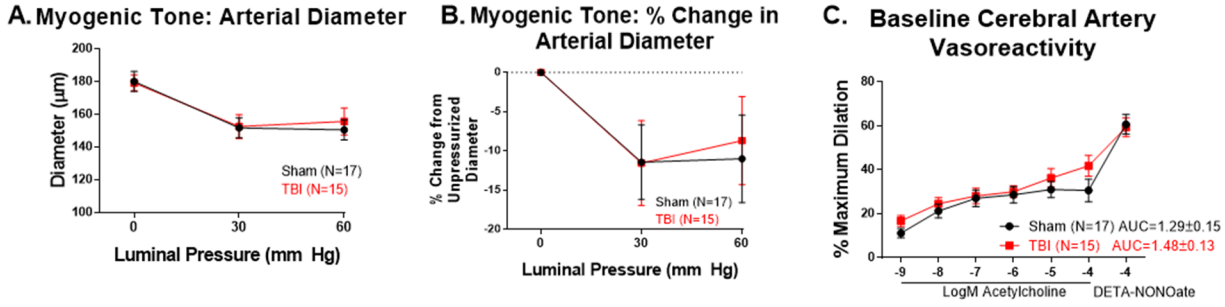


Fig. 4. Myogenic tone and baseline vasoreactivity. A-B. There was no significant difference in response to increasing intraluminal pressure between TBI and sham rat cerebral arteries. C. Dilator responses to increasing doses of acetylcholine and DETA-NONOate were also not significantly different between TBI and sham rats.

These results inform us that unstimulated, resting endothelial and smooth muscle cerebral arterial function is not impacted by mild TBI at 6 months but results following agonist stimulation with vasoactive stressors (see below) indicate that reliance only in resting, unstimulated arterial function may not be sufficient to uncover chronic vascular functional change.

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

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 above. Note that the false discovery rate values of ~0.1 is an acceptable threshold for initial screening of genes for future validation.

Laser captured (LCM) vessels from the Ca1 of the hippocampus were analyzed (DESeq2) in Sham and TBI rats at 6 mo. of age. Initial screening of the differentially expressed transcripts of TBI vs. Sham (Fig. 5A) revealed a list of genes often described as being associated with the process of neurodegeneration. One of the more significantly affected genes was plectin (Plec). Plec links the cytoskeleton to junctions found in the plasma membrane and plays an important role in maintaining the mechanical integrity and viscoelastic properties of tissues. Among other top hits were heat shock proteins, normally produced by cells in response to exposure to stressful conditions. Pathway analysis of the significantly upregulated genes (Fig. 5B) revealed:

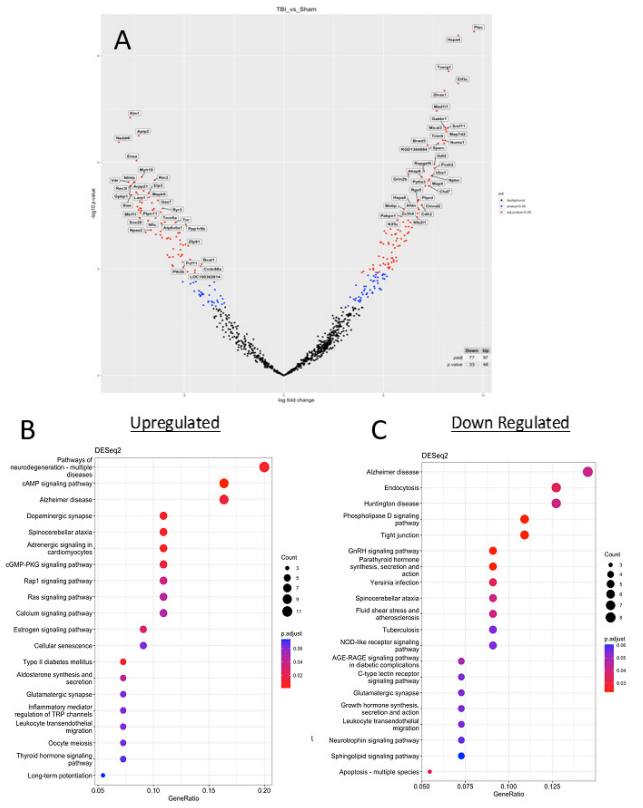


Fig. 5. A. Volcano plot of LCM CA1 vessels, sham versus mTBI. Significance is plotted versus fold-change on the y and x axes respectively. Pathway analyses of significantly upregulated genes (B) show top pathway being neurodegeneration-multiple-diseases, cAMP and Alzheimer's disease. Pathway analyses of downregulated genes (C) also showed significant association with Alzheimer's disease.

pathways of neurodegeneration-multiple-diseases, followed by cyclic AMP signaling and Alzheimer's disease. Pathway analysis of downregulated genes (Figure 5CB) also showed a significant association with Alzheimer's disease. These data indicate that TBI and neurodegeneration share overlapping biological changes, but what makes this very interesting is that most studies in Alzheimer's disease focus on neuronal health, and here we present data from vascular health, which may merit further investigation into Alzheimer's disease.

To evaluate neuroinflammation, we quantified activated astrocytes using GFAP staining and found no significant difference between sham and mTBI. There was also no significant difference between sham and mTBI in terms of markers of vascular inflammation as pial artery phosphorylated NFkB and RAGE were similar (Figure 6 D-E). Surprisingly, when we looked at BDNF expression, there was higher level in mTBI versus sham. The physiologic implication of this finding remains to be explored.

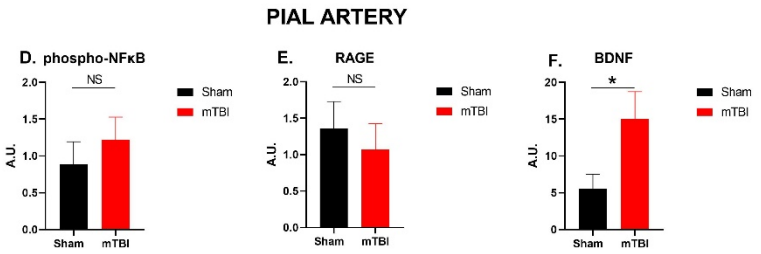


Figure 6. Tissue immunohistochemistry/immunofluorescence. Pial artery levels of phospho-NFkB and RAGE were not different. Surprisingly and unexpectedly, BDNF protein expression was higher in mTBI versus sham.

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.

Accomplishments: So far, we have not seen any difference in total antioxidant capacity between TBI and sham. We performed multi-cytokine assay of blood samples (N=10 each of TBI and sham). Our data show that at 6 months post-injury, there was significant elevation in IL-12 in TBI versus sham rats, with similar trend (although not statistically significant) with IL-5, IL-10 and TNF-a. This is an exciting finding as previous study implicated IL-12 abnormality in executive impairment post-acute TBI (https://www.liebertpub.com/doi/full/10.1089/neu.2016.4813) and our study suggests chronic impairment in this cytokine. IL-12 is produced by activated antigen-presenting cells (e.g. such as macrophages), promotes the development of Th1 responses and is an inducer of IFN-g production by T and NK cells. These findings can support new proposals to explore the hypotheses.

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

Accomplishments: Arteries from TBI and sham rats were processed and data collected. 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 vessels from TBI rats when exposed to Aβ42.

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

To sum, we found no significant difference in arterial smoothelin (mean±SD: 20.2±20% versus 21.0±20%, respectively, p=NS). We used semiquantitative means of measuring cerebral artery phospho-NFkB and RAGE protein expression using immunohistochemistry. So far, we found no significant difference in p-NFkB (1.60±1.1 vs. 1.10±1.2 AU TBI vs sham, p=0.3) or RAGE (0.96±1.1 vs. 1.5±1.3, p=0.3).

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 the LPS pretreated cohorts in terms of cognitive function, MRI imaging and vasoreactivity assessments. As detailed in prior reports, contrary to our hypothesis that LPS pretreatment would prime protective "hormesis"-type response in TBI, LPS pretreatment led to worse cognitive function. 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 in future proposals.

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: As discussed above, we showed no significant difference in resting baseline cerebral arterial dilator response to acetylcholine (endothelium-dependent function) and to DETA-NONOate (smooth muscle-dependent function). Following arterial exposure to vascular agonists

known to impair vascular function (Aβ42, high glucose HG or angiotensin II), there was no difference in post-agonist dilator response between mTBI and sham rats following exposure to Aβ42 or HG (Figure 7 B1-3 and C1-3). However, there was significant difference between mTBI and sham following exposure to angiotensin II (Figure 7 A1-3), a key hormone in inducing hypertensive state. We believe this is a key finding in that even though there is recovery of cerebrovascular function 6 months following mTBI in the resting state, there may be persistent subtle vascular perturbation that could be unmasked in the presence of vascular stressor such as angiotensin II, suggesting normal resting but possible impaired vascular reserve.

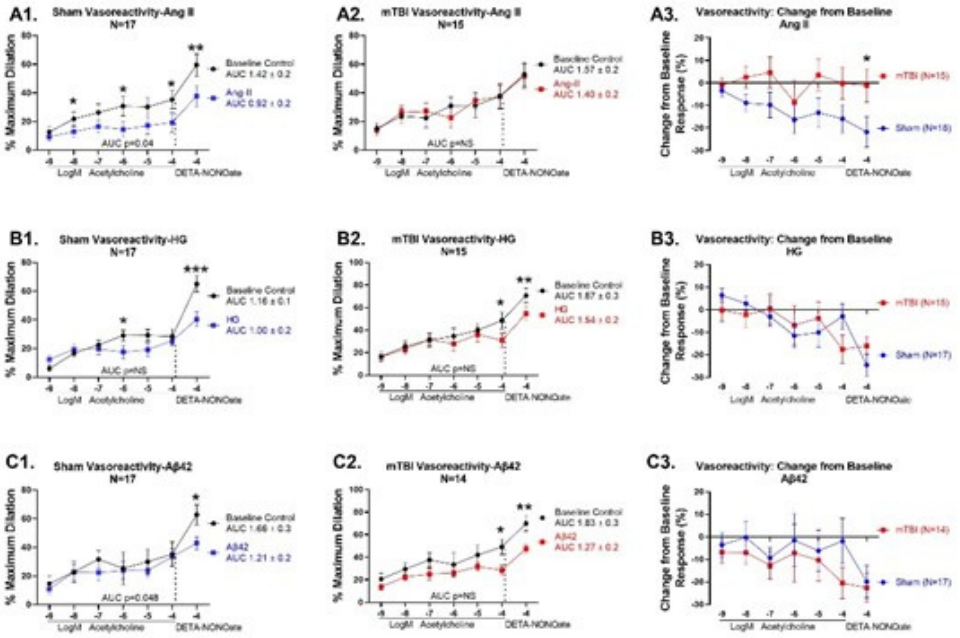


Fig. 7. Cerebral artery dilator responses at 6 months following exposure to vascular agonists. A. Sham rats showed reduced dilator response to acetylcholine and DETA-NONOate following exposure to angiotensin II (A1), which was not seen in mTBI rats (A2). There was significant reduction in dilation to DETA-NONOate in sham compared to mTBI (A3). B. Overall dilator response to acetylcholine (AUC) was not different following high glucose exposure in both sham and mTBI, while dilation to DETA-NONOate was reduced in both sham and mTBI (B1-B2). There was no difference in change in dilator response to high glucose between sham and mTBI (B3). C. There was marginal reduction in overall dilator response to acetylcholine (AUC) following Aβ42 in sham but not in mTBI, but dilation to DETA-NONOate was significantly reduced in both sham and mTBI (C1-2). There was no difference in change in dilator response to Aβ42 between sham and mTBI (C3).

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. Our data on cognitive and vascular function were detailed in last annual and quarterly reports. Although there was a trend towards worse NOL and TOR scores in STZ treated rats, 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 (P=0.002) and smooth muscle-dependent function (P<0.001) in STZ-treated rats. The interaction term for TBI x STZ exposure was not significant.

2-way ANOVA analyses on arterial responses following exposure to Aβ, high glucose or angiotensin-II in this cohort. In this larger cohort of rats as compared to the data reflected in Figure 7, we again showed that TBI resulted in altered dilator response (endothelium and smooth muscle dependent) when compared to sham following exposure to angiotensin-II, but STZ treatment showed borderline (p=0.06) significant difference in endothelium-dependent dilation (based on area under the curve) following angiotensin-II exposure. In this response, there was significant interaction term between TBI and STZ (p=0.04) suggesting that later development of diabetes in the setting of TBI may alter vasoreactivity related to angiotensin-II signaling. 2-way ANOVA also revealed that dilator response to acetylcholine (endothelial function) following exposure to Aβ42 is altered in TBI versus sham, but not with STZ treatment, while smooth muscle function response post-Aβ42 was altered by STZ treatment but not by TBI. Further mechanistic investigations are needed to explore the physiological implications of these findings. Vasoreactivity following exposure to high glucose was not altered by TBI or STZ treatment, unlike angiotensin II or Aβ42.

We are completing immunohistopathologic and immunofluorescence evaluation of brains and cerebral artery tissue.

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

The Translational Neurotrauma Research Program used to host 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. Due to COVID-19, there was suspension of the journal club, and the opportunity for online discussions exist. At this time, this training opportunity has been suspended.

Lifshitz and Migrino Lab: Ms. Hannah Emerson is a laboratory technician recruit who took over the responsibilities of Mr. Conor Young. Ms. Emerson was trained on animal handling, animal care, animal injections, tissue collection, Western blotting as well as planning of experiments. She was accepted to U of Arizona Veterinary Med School and will be able to use her training for this career path. Mr. Connor Leighty is a Masters student who took over Ms. Emerson's responsibilities as part-time lab personnel and is learning optimization and processing of tissue histopathology.

How were the results disseminated to communities of interest?

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. A poster was presented at the National Neurotrauma Society annual symposium held online July 2021. Two abstracts were presented for presentation at the 2021 Arizona Alzheimer's consortium scientific meeting, the largest scientific meeting in Arizona dealing with neurodegenerative conditions. Our manuscript was accepted for publication at the J Neurotrauma PMID 35593008.

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

We will complete brain tissue and cerebral arterial processing for IHC analyses. Once we have the histopathology results, we will perform multivariable analyses (TBI vs. sham, LPS vs. vehicle, STZ versus vehicle, cognitive function/imaging/vasoreactivity/pathology outcome measures) for a comprehensive assessment of the chronic effects of TBI and the modulating factors, with focus on assessing interrelationships. We plan on completing additional manuscripts out of these analyses.

4. **IMPACT:**

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

The major impact of our project is the validation and characterization of this model of TBI, phenotypically classifiable as "mild" or non-severe. This classification is based primarily on the lack of overt neuropathologic changes, with chronic cognitive and vascular changes. Thus, we empirically show that even "mild" TBI could have medium-term pathophysiologic consequences. Although this observation was suggested by retrospective studies of human injuries, our results were the first comprehensive empirical evidence of the chronic effects of mild TBI on cognitive and vascular function that we are aware. This would form an empiric basis for rigorous prospective evaluations of soldiers, Veterans or civilians exposed to mild or moderate TBI or concussions to re-assess risk of future cognitive and vascular abnormalities, specifically when vascular challenges are introduced. Our results validate the importance of the midline fluid percussion 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. Contrary to our hypothesis, the diabetes model we selected (STZ treatment) did not have additive or synergistic effects on cognitive function or in vivo cerebral blood flow effects to TBI, although some interactions were noted between TBI and STZ treatment in altering endothelium-dependent dilator response post-angiotensin II. Future studies will need to determine if other models of diabetes (e.g. animal models more closely mimicking type 2 diabetes) will or will not show interaction with TBI. The mechanistic pathway signaling from our laser microdissected RNAseq analyses (pending full analyses) will hopefully provide additional known or new pathways to pursue to identify potential druggable targets. We are hopeful that completing the comprehensive assessment of functional, structural and pathological outcomes would provide additional mechanistic insights.

What was the impact on other disciplines?

This collaborative effort from various disciplines (neuroscience, cardiovascular disease, imaging) allowed a comprehensive evaluation of the medium-term consequences of mild TBI on multiple modalities that have previously not been measured simultaneously in the same cohort. Thus we will have the ability to tease out associational relationships among cognitive, vascular, imaging, genomic, functional, gross structural and microscopic outcomes of TBI. This framework serves as a practical foundational approach to expand on the findings of this project.

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:

Nothing to report

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

1. When we analyzed our synaptophysin immunohistochemistry images using standard processing, PI Dr. Lifshitz noted wide variability in staining conditions among different batches of tissues, raising rigor concerns about comparability of measured tissue signals. While we also attempted to be rigorous and consistent with our brain bregma selection, there was also concern about variability in regions selected. Out of these concerns, we decided to go back to our paraffin embedded brain sections and redo some of the IHC staining, measurement and analyses informed from these prior experiences.
2. We had personnel turnovers including the departure of Mr. Seth Truran for a private sector job and Ms. Hannah Emerson for Veterinary Medicine school. Each of them had specific expertise and roles in the tissue processing and evaluation. Their roles were replaced by additional personnel but this required additional training for these tasks.

Changes that had a significant impact on expenditures

The full cost of the RNAseq of microglia and vessels was not fully anticipated so we had to make a decision on the practical number of samples to assay.

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

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

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

  Journal publications. Griffiths D, Law L, Young C, Fuentes A, Truran S, Karamanova N, Bell LC, Turner G, Emerson H, Mastroeni D, Gonzales R, Reaven PD, Quarles CC, Migrino RQ, Lifshitz J. Chronic cognitive and cerebrovascular function following mild traumatic brain injury in rats. J Neurotrauma 2022 May 20. Online ahead of print. PMID 35593008

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

Abstract presented "Experimental TBI induces long term cognitive deficits and vascular pathology" at the 2020 15th Annual NABIS Conference on Brain Injury. Presented poster presentation 2021 National Neurotrauma Society Symposium (online) Griffiths, DR, LM Law, N Karamanova, S Truran, LC Bell, GH Turner, C Quarles, RQ Migrino, J Lifshitz. (2021) "Experimental Brain Injury Induces Long Term Cognitive Deficits and Vascular Pathology." J Neurotrauma https://doi.org/10.1089/neu.2021.29111.abstracts Poster presentation 2021 Arizona Alzheimer's Consortium scientific session: "Association between cerebrovascular function and chronic cognitive dysfunction following mild-moderate traumatic brain injury in rats and lack of modulating influence by diabetes."

Poster presentation 2021 Arizona Alzheimer's Consortium scientific session: "Chronic pial cerebral arterial function and cognitive function following mild-moderate traumatic brain injury in rats".

Dr. Lifshitz lecture: Department of Medicine, Banner University Medical Center - Phoenix 5/13/2022: *Innovation in Translational Neurotrauma Research*

Other publications, conference papers and presentations.

In light of DOD grant support which provided Dr. Migrino and Dr. Lifshitz protected research time and allowed continued employment of research personnel, 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.

4. Ahmad S, **Truran S, Karamanova N**, Kindelin A, Lozoya M, Weissig V, **Griffiths D**, Vail T, **Lifshitz J**, Ducruet AF, **Migrino RQ**. Nanoliposomes reduce stroke injury following middle cerebral artery occlusion in mice. Preprint DOI: 10.20944/preprints202108.0302.v1. Under review in International Journal of Molecular Sciences journal.

5. YV Doust, RK Rowe, PD Adelson, **J Lifshitz**, Ziebell, JM. (2021) Age-at-Injury Determines the Extent of Long-Term Neuropathology and Microgliosis after a Diffuse Brain Injury in Male Rats. *Frontiers in Neurology* 12:722526.

6. Beitchman, JA*, **J Lifshitz***, NG Harris, TC Thomas, AD Lafrenaye, A Hånell, CE Dixon, JT Povlishock, RK Rowe. (2021) Intracranial Mechanics of Fluid Percussion Brain Injury in the Rodent. *Neurotrauma Reports* 2(1):59-75

- **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?

Name: Seth Truran
Project Role: Research Associate
Nearest person month worked: 0
Contribution to Project: Mr. Truran assisted team members as needed.

Name: Nina Karamanova DVM
Project Role: Research Associate
Nearest person month worked: 4
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: Hannah Emerson
Project Role: Animal and Laboratory Technician
Nearest person month worked: 0
Contribution to Project: Ms. Emerson assisted team members as needed.

Name: Robert Schaefer
Project Role: Laboratory Technician
Nearest person month worked; 2.5
Contribution to Project: Assisted in imaging of tissue slides, optimization and analyses

Name: Connor Leighty
Project Role: Laboratory Technician
Nearest person month worked; 3
Contribution to Project: Assisted in planning and optimization of tissue slides

Name: Gail Farrell
Project Role: Research Coordinator-Backup
Nearest person month worked: 0
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
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
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

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: Chengcheng Hu
Project Role: Investigator
Nearest person month worked: 0
Contribution to Project: Dr. Hu is the biostatistician on the project, with input on the study design, data organization, and analytical modeling.

Name: Raymond Migrino MD
Project Role: Joint PI
Nearest person month worked: 0.6
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: 0.3
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
Contribution to Project: Dr. Gonzales helped optimize protocols and methodology as well as data interpretation.

Name: C. Chad Quarles, PhD
Project Role: Co-investigator
Nearest person month worked: 0
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
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 0

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: 0.6
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: 0
Contribution to project: Methodologic optimization of vascular laser capture microdissection procedure and tissue sectioning and processing.

Name: Laura Bell PhD
Project Role: Consultant
Contribution to project: Dr. Bell assisted Dr. Quarles in completing the cerebral blood flow analysis of MRI data (data presented in Figures 2-3 in this report).

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

Migrino was awarded an NIH R21 (2022-2024)

What other organizations were involved as partners?

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: QUAD CHARTS:

9. APPENDICES:

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	95%
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	100%
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	100%
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	95%
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	100%
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	95%
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	100%
Milestone(s) Achieved: Determine whether ex vivo cerebral vessels isolated from injured rats have worse endothelial function when exposed to high	20	100%

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	95%
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	95%

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

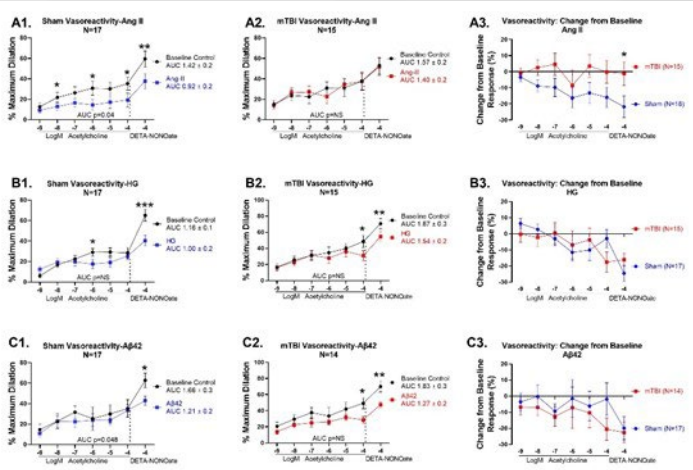
1A. Compare 6-month cerebral flow, and ***ex-vivo*** function of cerebral arteries from TBI versus uninjured rats and determine the relationship between vascular function with cognitive function.

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.



Post 6 month sacrifice cerebral artery dilator artery responses following exposure to vascular agonists showing differences in response between sham and mTBI with angiotensin II (top), but not high glucose (middle) or Aβ42 or high glucose.

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: 07/31/2022

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
- Continuation of histologic examinations and multivariable analyses

Budget Expenditure to Date: July 31, 2021

Projected Expenditure: $1,300,000

Actual Expenditure: $1,212,632.00