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TITLE: Targeting the Endotheliopathy of Trauma in Hemorrhagic Shock and Traumatic Brain Injury with Freeze-Dried Platelets

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CONTRACTING ORGANIZATION: University of California, San Francisco, CA

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13. SUPPLEMENTARY NOTES

AA. ABSARACA

The goal of this project is to test the therapeutic potential of freeze dried platelets-FDPlts (Thrombosomes)- in disease conditions characterized by 1) inflammation, 2) vascular instability and 3) coagulation disturbances, which are all components of the endotheliopathy of trauma (EOT) (refs). Aside from their hemostatic properties, this proposal aims to also determine the mechanisms of action of the freeze dried platelets (FDPlts (Thrombosomes)) on the EOT in traumatic brain injury (TBI) and shock induced acute lung injury (ALI); all conditions with few if any effective treatment options.
We hypothesize that FDPlts (Thrombosomes) will have potent hemostatic properties comparable to fresh platelets and that they will attenuate and mitigate the endotheliopathy of trauma (EOT) in TBI and HS induced ALI. We hypothesize that FDPlts (Thrombosomes) can be used as a stand-alone early therapy to mitigate outcomes in trauma.

15. SUBJECT TERMS

TBI- Traumatic brain Injury
HS- Hemorrhagic Shock, ALI- Acute Lung Injury

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1. INTRODUCTION:

Currently in blood-banking practice in the US, platelets are stored in incubators at 22°C, with gentle agitation for up to 5 days. The main reason for this practice of storage at 22°C is to allow for adequate circulating numbers of platelets post transfusion and to avoid the risk of bacterial contamination. It has been shown that storage of platelets at 22°C for 5 days is associated with a decline in function of the platelets, also known as a storage lesion. One option is for blood banks to store platelets at 4°C, which is currently approved for 3 days of storage; however, diminished function of 4°C platelets has also been reported. Alternatively, a freeze-dried platelet (FDPlts) product can circumvent these challenges by providing hemostasis, prolonging the shelf life of platelets without cold storage and significantly enhancing the utilization and safety of transfused platelet units. FDPlts (Thrombosomes), made by Cellphire Inc., are an infusible freeze-dried platelet-derived hemostatic agent, stabilized with trehalose and polysucrose prior to and during freeze-drying. They can be stored at room temperature with prolonged shelf life (>1 yr), eliminating the need for bacterial testing, and logistically allow for platelet availability in remote and austere conditions.

Characterization studies demonstrate that FDPlts (Thrombosomes) express markers such as P-selectin and phosphatidylserine, hence indicating that they are activated. FDPlts (Thrombosomes) have demonstrated to have potent hemostatic properties. Canines undergoing coronary artery bypass grafting (CABG) treated with fresh platelets or FDPlts (Thrombosomes) showed a dose dependent decrease in blood loss. FDPlts (Thrombosomes) also deliver hemostatic efficacy in uncontrolled arterial bleeding in rats and New Zealand white rabbits (NZWR) with busulfan induced thrombocytopenia. Thus, FDPlts (Thrombosomes) are primed hemostatic agents that can be used towards the treatment of acute uncontrolled hemorrhage in bleeding patients. Safety studies with FDPlts (Thrombosomes) have been performed in several species including non-human primates and humans. No evidence of systemic thrombosis or non-specific thrombosis has been noted, which is a concern when utilizing an activated platelet product. **The goal of this project is to test the therapeutic potential of freeze dried platelets-FDPlts (Thrombosomes)-in disease conditions characterized by 1) inflammation, 2) vascular instability and 3) coagulation disturbances, which are all components of the endotheliopathy of trauma (EOT) (refs).** Aside from their hemostatic properties, this proposal aims to also determine the mechanisms of action of the freeze dried platelets (FDPlts (Thrombosomes)) on the EOT in traumatic brain injury (TBI) and shock induced acute lung injury (ALI); all conditions with few if any effective treatment options.

We hypothesize that FDPlts (Thrombosomes) will have potent hemostatic properties comparable to fresh platelets and that they will attenuate and mitigate the endotheliopathy of trauma (EOT) in TBI and HS induced ALI. We hypothesize that **FDPlts (Thrombosomes) can be used as a stand-alone early therapy** to mitigate outcomes in trauma.

2. KEYWORDS:

Hemorrhagic shock, Freeze-dried platelets, Thrombosomes, Traumatic brain injury, Inflammation, Vascular dysfunction, endotheliopathy of trauma

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Characterize *in vitro* effects of Thrombosomes	**Months**	**UCSF (Pati)**
Major Task 1: Months 1-6: Obtain approval from institutional IACUC and ACURO for HS model.	100% complete	Dr. Pati
Milestone(s) Achieved: ACURO approval for HS and TBI model completed	6	
Major Task 2: FDP-Thrombosomes effects on endothelial permeability and signaling (Aim 1)		Dr. Pati
Subtask 1: Grow PECs (pulmonary endothelial cells) and brain endothelial cells (BECS) to sufficient quantities for *in vitro* assays Start ECIS and endothelial functional assays of platelet groups	3-6	100% completed
Subtask 2: Complete ECIS assays of platelet groups on all endothelial cells. Conduct Western Blots of endothelial signaling pathways and staining of ECs for junctional and cytoskeletal markers	7-9	100% completed
Subtask 3: Continue and complete Western Blots of endothelial signaling pathways and staining of endothelial cells for junctional markers.	10-12	
Milestone(s) Achieved: Comparison of Platelet groups on EC permeability and PEC and BEC signaling	12	
Major Task 3: Effect of FDP (Thrombosomes) on HS induced ALI- 3 hour model (Aim 2)-		Dr. Pati
Subtask 1: HS induced ALI Model acute three hour study (51 animals)	6-9	100% completed
Subtask 2: HS induced ALI Model acute three hour study (51 animals)	10-12	
Subtask 3: Sectioning of HS induced ALI 3 hour Model (102 animals)	13-15	100% completed
Subtask 4: Tissue analysis 3 hour HS model (102 animals)	16-18	100% completed
Milestone(s) Achieved: Determine efficacy and optimal dose of	18	

FDP in vivo to modulate EOT/ALI in 3 hour model of HS		
Major Task 4 Effect of FDP (Thrombosomes) on HS induced ALI-24 hour model (Aim 2)		Dr. Pati
Subtask 1: HS induced ALI Model 24 hour study (84 animals)	19-24	100% completed
Subtask 2: Sectioning of HS induced ALI 24 hour Model (84 animals)	25-27	100% completed
Subtask 3: Tissue analysis 24 hour HS model (84 animals)	28-30	100% completed
Milestone(s) Achieved: Determine efficacy and optimal dose of FDP in vivo to modulate EOT/ALI in 24 hour model of HS	27	
Major Task 5 Effect of FDP (Thrombosomes) in TBI (Aim 3)		Dr. Pati
Subtask 1 TBI – optimizing dose of FDP (perform surgeries and collect tissue at 3 day time point) – Total of 80 mice	13-16	100% completed
Subtask 2 - Tissue analysis (barrier permeability – 80 mice)	16-18	100% completed
Subtask 3 TBI – optimizing timing of delivery of FDP (perform surgeries and collect tissue at 1 day and 3 day time point) – Total of 70 mice	18-21	
Subtask 4 - Tissue analysis (sectioning and staining – 70 mice)	19-24	100% completed
Subtask 5 – Setting up behavior tests with control age and strain matched mice	18-24	100% completed
Subtask 6 –Behavior test optimization – data analysis	24-30	40% completed
Subtask 7 - TBI – Acute time point surgeries and tissue collection (168 mice)	25-31	
Subtask 8 TBI – Acute time point tissue sectioning, staining, and analysis (168 mice)	31-36	
Subtask 9 TBI – chronic time point surgeries and tissue collection, sectioning, staining (115 mice)	35-42	
Subtask 10 TBI – tissue analysis and behavior analysis for chronic time point mice (115 mice) and overall data analysis	40-48	
Milestone(s) Achieved: Complete studies of FDP effects in TBI.	48	
Major Task 6: Measure Thrombosome effects on endothelial glycocalyx and clot formation		Dr. Pati
Intravital Microscopy of mice (64 animals)	31-36	60% completed
Intravital Microscopy of mice (64 animals)	37-42	
Milestone(s) Achieved: Completion of testing for FDP effects on endothelial glycocalyx and clot formation	36	
Milestone(s) Achieved: Thrombosome production		
Major Task 7: Submit abstracts to meetings and manuscripts. Submit final report to DOD	48	Dr. Pati

What was accomplished under these goals?

The major work completed during this time was as follows:

1. *We got our triennial IACUC protocol approved (4-12-2023) and have approval from ACURO to continue with our experiments.*
2. *We have continued studies on traumatic brain injury (TBI) model. Last year, we focused on the long-term behavior outcomes after TBI. We were able to generate the mice (sham vs. TBI) and perform the behavior tests at 4-6 weeks post-injury. All mice have been euthanized and brains processed for histological evaluation.*
3.

Methods:

Traumatic Brain Injury (TBI) model

A controlled cortical impact (CCI) injury was performed on 10-12 week old C57Bl/6J mice (Jackson Laboratories, Sacramento, CA). Craniotomy (5mm) was performed on isoflurane-anesthetized mice and a moderate level of cortical contusion injury was performed using stereotactic device, Impactor 1 (Leica Biosystems, Buffalo Grove, IL). A 3mm piston impacted the cortex at a velocity of 4.5 m/s, depth of 1.2 mm, and dwell time of 300 ms. Animals were generated for long-term behavior outcomes. Sham mice were mice that received anesthesia and placement on stereotactic device, with incision but no craniotomy. Animals were randomized to receive injury or sham surgery. At the end of the behavior testing, mice were euthanized and brains were collected for histological analysis.

Behavior Tests

All behavior tests were performed in a blinded manner and animals were randomized. Tests began at 4 weeks post-injury and continues for two weeks.

Motor Behavior Testing

Rotarod: The purpose of this test is to determine a mouse's sensorimotor skills (motor coordination and balance) and motor learning. To do this, the mice are placed on a rod that is rotating (similar to log-rolling) and the mouse must stay on the rod. The rod will turn faster and faster until the mouse falls off. Each mouse gets a total of 9 trials (3 trials per day for 3 days). There is a 15-20 minute inter trial breaks. The total duration per trial is 300 seconds (5 minutes), with a start speed 5 RPM, with increments and going to max speed of 50 RPM. The duration of each mouse on the rotarod per trial is noted.

Open Field Test: Open field is a test used to determine an animal's overall general activity levels, gross locomotor function and anxiety levels (Thigmotaxis). The basic principle is that a more anxious animal will spend more time around the periphery of an open arena and less time exploring the center. Animals are placed in an open arena (16"x16") for 10 minutes and movement track is recorded using Noldus camera tracking and analysis software. From the data generated, we can analyze motor activity, measure how much time an animal spends in the center of the arena (8"x8") versus the periphery of the arena.

Elevated Plus Maze and Zero Maze: These tests are a measure of anxiety where the time spent in open area vs. closed area is measured.
Novel Object Recognition: This test is used to assess cognitive function (specifically learning and memory). This test is performed over three days and time spent with familiar vs. novel objects is measured. Mice have an innate preference for novelty so the recognition of familiar object is the measure of hippocampal dependent memory.
Trace Fear Conditioning: This test is used to measure hippocampal dependent memory and is complimentary to the Novel object recognition test described above. In this test the mice are exposed in a paired fashion to a noxious stimulus (shock) and neutral stimulus (tone). Through repeated conditioning, the neutral stimulus becomes the conditioning stimulus. The time of freezing on exposure to the neutral stimulus or the chamber is measured as assessment of memory.

Brain Intravital Imaging for tracking thrombosomes
Mice are initially anesthetized with a combination of Ketamine and Xylazine and placed on a warmed operating table. The depth of anesthesia is tested with a paw pinch at which point the trachea is then isolated and cannulated with PE-90 tubing to facilitate mechanical ventilation with a rodent ventilator at which point Isoflurane is continuously delivered at 1-2% in oxygen for maintenance anesthesia. The mice are then placed in the prone position. The skin (2cm x 2cm) on top of the skull is carefully removed to expose the brain. Hemostasis is achieved with pressure using Qtips. An incision is made in the neck to expose the jugular vein and a PE-10 tubing is inserted into the jugular vein and positioned still to allow for injections of tagged thrombosomes. We then place a custom training imaging window on top of the craniotomy window. A microscope objective is then lowered onto the brain imaging window to facilitate imaging for up to 2 hours at the end of the imaging session the mice are euthanized. FITC-tagged thrombosomes were resuspended in sterile water and injected into the mice through the jugular vein during imaging.

Statistical analyses
For motor analysis on rotarod, the data is analyzed by RM 2way ANOVA and for motor learning, we calculate the delta between day 3 and day 1 and comparison between he two groups is made by unpaired two tailed t-test. For motor and anxiety analysis in open field and mazes, the data is analyzed by unpaired two tailed t-test.

Results:

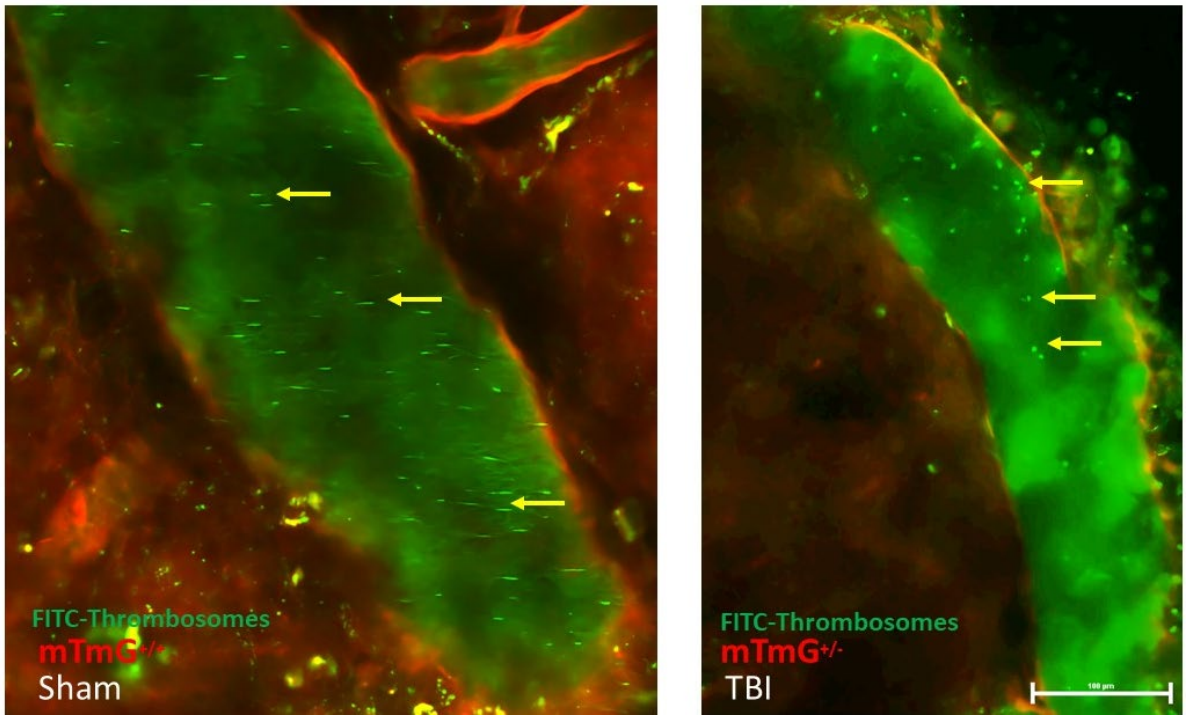

Figure 1 Representative images of FITC tagged Thrombosomes (green) in the blood vessels of sham (left) and injured mTmG mice (right). Tissue and blood vessel lining is in red and FITC tagged thrombosomes are in green. Free flowing tagged thrombosomes are seen in both the animals, suggesting that the thrombosomes are in circulation and cross the blood brain barrier. In the injured animal, there is release of the thrombosomes (yellow arrows in both parts) in the parenchyma from the blood vessel.

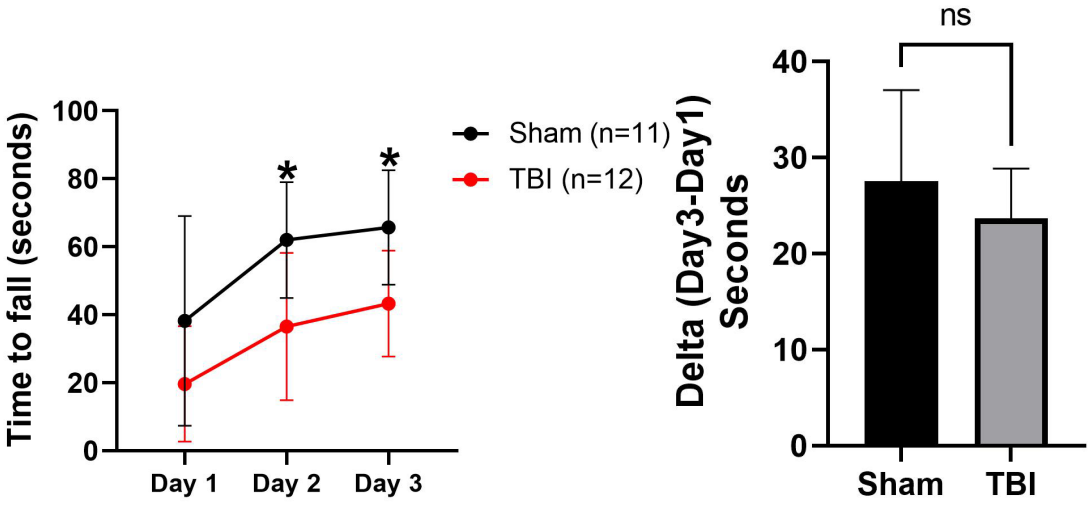

Figure 2: Establishing baseline of model: Mice that have sustained TBI display motor deficits but have no motor learning deficits. Graph on left shows that mice in both the groups improve over time, (RM two-way ANOVA, no significant interaction, effect of time ($p<0.0001$), Sham mice stay longer on the rod on days 2 and 3 as compared to day 1 ($p<0.01$ and $p<0.001$ respectively). Even though TBI mice stay longer on days 2 and 3 as compared to day 1, their slope is different ($p<0.05$ and $p<0.01$ respectively). There is also a significant effect of injury ($p=0.0023$), with sham mice performing better as compared to TBI mice on days 2 and 3 ($p<0.05$), as indicated on the graph. Thus, implying that the TBI mice display loss of motor coordination and balance after injury. Motor learning as measured by improvement between day 3 and day 1 (right graph) showed that there was no difference between groups. columns represent mean±SD; sham (n=11) TBI (n=12).

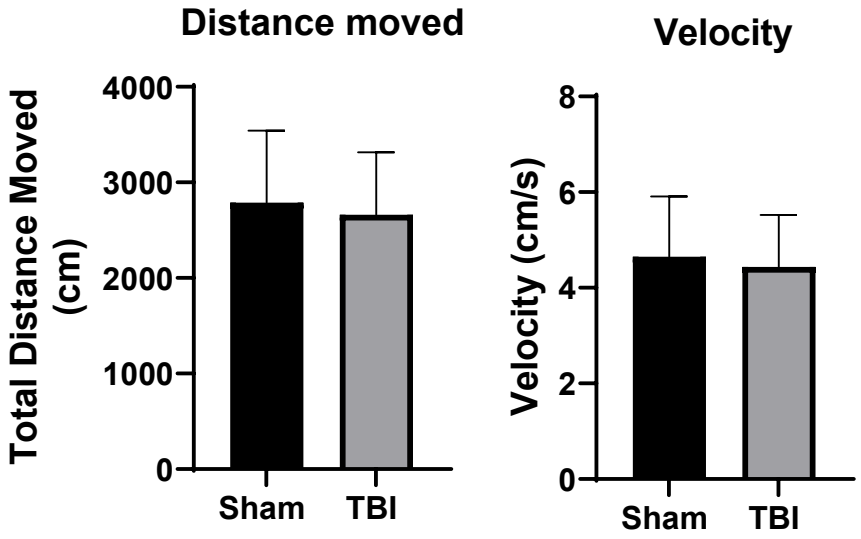

Figure 3: Establishing baseline of model: Mice that have sustained TBI display no overall difference in total activity and gross motor function. Graph on left shows total distance moved, as a measure of overall motor activity. Graph on the right depicts the speed with which the mice move. There was no difference between the groups for both the measurements, columns represent mean±SD; unpaired two-tailed t-test ($p>0.05$), sham (n=11) TBI (n=12).

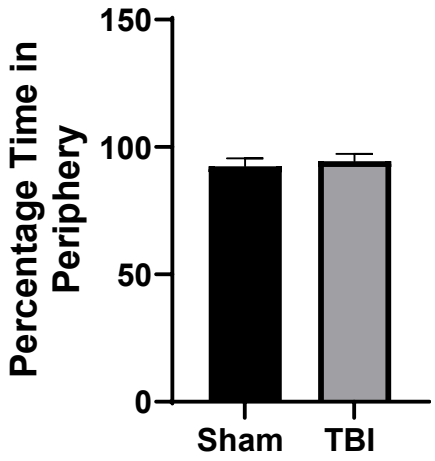

Figure 4: Establishing baseline of model: Mice that have sustained TBI display no overall difference in level of anxiety as measured by time spent in the periphery of the open field (Thigmotaxis). Graph shows percentage time spent in the periphery, as a measure of overall anxiety level. There was no difference between the groups for both the measurements, columns represent mean±SD; unpaired two-tailed t-test ($p>0.05$), sham (n=11) TBI (n=11).

The baseline behavior function of the TBI model is still a work in progress and we still have to analyze the data from the context discrimination and the other forms of anxiety measurements where we anticipate we will see differences.

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

Lindsay Vivona, the technician in our laboratory that was trained started graduate school studies.

Byron Miyazawa was trained to set up behavioral experiments.

Callie Keane has started in the laboratory and is learning figure generation.

How were the results disseminated to communities of interest?

Nothing to report.

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

- We will continue to analyze data from behavior test.
- We will proceed with our behavior studies with Thrombosomes
- We will continue the intravital microscopy studies
- We will write up a manuscript on acute effects of thrombosomes after TBI

4. **IMPACT:**

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

- We developed methodology of intravital imaging of brain to track the entry of the thrombosomes in the brain vasculature after injury. This has implications in other studies of therapeutic tracking.
- Also, the methodology as a whole can be used to visualize changes in vasculature after injury/disease, such as, but not limited to perfusion status, vascular leak and inflammation.
- Our preliminary behavior analysis shows no deficits in motor coordination and balance at a month following injury, however these are still in progress.

What was the impact on other disciplines?

The use of freeze dried platelets could be of great utility for storage and availability of platelets in remote and austere environments and also be a safer alternative from a infectious stand point of bacterial contamination. Eventually this could change Blood Banking practice in the US and military settings.

What was the impact on technology transfer?

Based on the data that we generated, Cellphire will be gearing up to run clinical trials for utility of Thrombosomes in traumatic brain inured individuals.

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

We have requested a 1 year NCE to complete the remaining behavior and intravital studies.

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

Nothing to report

Changes that had a significant impact on expenditures

Nothing to Report

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

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

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

Journal publications.

Barry M, Trivedi A, Vivona LR, Chui J, Pathipati P, Miyazawa B, Pati S. RECOVERY OF ENDOTHELIOPATHY AT 24 HOURS IN AN ESTABLISHED MOUSE MODEL OF HEMORRHAGIC SHOCK AND TRAUMA. Shock. 2022 Oct 1;58(4):313-320. doi: 10.1097/SHK.0000000000001984. Epub 2022 Aug 26. PMID: 36256627.

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

Nothing to Report

Other publications, conference papers and presentations.

April, 2022- presented data at Pediatric meeting on Transfusion Med at University of Alabama
October 2022- presented at Heretic meeting UPMC Pittsburgh, PA
May, 2022- presented at CTTACC in Scottsdale, AZ

- **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: Shibani Pati MD PhD
Project Role: Principal Investigator
Nearest person month worked: 2.4 cal months
Contribution to Project: Dr.Pati is the principal investigator on this grant and has been overseeing the planning, data analysis and execution of the entire grant.

Name: Alpa Mahuvakar PhD.
Project Role: Research Scientist
Nearest person month worked: 6.0 cal months
Contribution to Project: Dr. Mahuvakar is a co-investigator and is involved in planning of studies, analysis of data, running/execution of all research experiments, including in vitro endothelial cell signaling and traumatic brain injury.

Name: Byron Miyazawa
Project Role: Research Associate
Nearest person month worked: 6.0 cal months
Contribution to Project: Running/execution of platelet assays, endothelial assays, staining of endothelium and signaling analysis

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

None

What other organizations were involved as partners?

Cellphire Rockville, MD
All Thrombosomes were provided by Cellphire

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS: *N/A*

9. **QUAD CHARTS:** *N/A*

10. **APPENDICES:** *N/A*