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14. ABSTRACT

Traumatic brain injury (TBI) is a significant cause of death and disability in military & civilian populations. Cell therapy with mesenchymal stem cells (MSCs) shows promise for TBI. Because not all MSCs are equivalent, further work is needed to define optimal MSC products to minimize risk and maximize benefit. Here we proposed to establish a panel of assays to evaluate safety and potency of cell therapy products prior to administration. Assay results were used to select MSCs with different profiles for preclinical testing in rats. The maximal tolerated dose of MSCs will be determined in both uninjured and injured rats, and the distribution and retention of labeled cells following intravenous administration will be determined. MSC administration will be tested in rats subjected to TBI, and the impact of the cell therapy will be measured using a combination of histological, functional and behavioral testing. Because the long term consequences of TBI can be devastating and negatively affect quality of life in survivors, any treatment that can reduce the amount of brain damage or enhance brain healing will be of tremendous importance

15. SUBJECT TERMS
Traumatic Brain Injury, Cell Therapy, Mesenchymal Stem/Stromal Cells

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

Traumatic brain injury (TBI) is a significant cause of death & disability in both military and civilian populations. Cell therapy with mesenchymal stem cells (MSCs) or other progenitors shows promise for TBI treatment and other types of brain injury, but further work is needed to define the optimal MSC products to minimize risk and maximize benefit. MSC products are not all equivalent in safety and potency, and different MSCs are likely to have different efficacy. Here we implement in vitro testing to evaluate both safety & potency of different MSCs, which will ultimately be validated using an animal model for rat TBI. Maximal tolerated dose of MSCs will be determined in both uninjured and severely injured rats, and the distribution and retention of labeled cells *in vivo* following IV administration will be determined. Finally, rats will be subjected to moderate TBI and the effects of MSC administration will be evaluated.

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

Traumatic brain injury, cell therapy, mesenchymal stem cells, coagulation, macrophage polarization, vascular permeability

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.

Aim 1. In vitro safety and potency testing of human MSCs. Tasks proposed include measuring pro-coagulant activity of MSCs using a variety of methods, evaluating cell surface TF expression and generating cells with TF knocked down (reduced) by siRNA/shRNA in order to determine if TF is actually necessary for pro-coagulant activity and/or in vitro potency in MSCs. To support these efforts, we also proposed to develop and implement assays to measure MSC effects on macrophage polarization and on endothelial permeability. (Estimated percentage of completion: 100%)

Aim 2. Animal testing. In this aim we will determine dose response for "good" and "bad" MSCs in uninjured and injured animals (rats). Biodistribution of labeled cells will be determined following administration. (Estimated percentage of completion: 50% (studies in uninjured animals completed but studies in injured animals delayed due to shutdown of our laboratories and animal facilities due to COVID-19 pandemic Expected completion date: Unclear; depends on date of re-opening of facilitis).

Aim 3. Testing of MSC administration for TBI. This goal is slated for the 3rd year of the proposal, and has not yet begun (0% completed).

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.

Aim 1. In vitro safety and potency testing of human MSCs. Tasks proposed included measuring the pro-coagulant activity of multiple MSCs using a variety of methods, evaluating cell surface TF expression and generating cells with TF knocked down (reduced) by siRNA/shRNA in order to determine if TF is actually necessary for pro-coagulant activity and/or in vitro potency in MSCs. To support these efforts, we also proposed to develop and implement assays to measure MSC effects on macrophage polarization and on endothelial permeability.

Tasks & Progress:

A. Measure pro-coagulant activity of multiple MSCs, including MSCs derived from umbilical cord.
   - This task was completed in the previous grant year and covered in the Year 1 annual report (April 2019). However, this type of testing will still be done on future preparations of cells being prepared for animal administration.

B. Determine cell surface TF expression by flow cytometry. Our previous work showed that there was a good correlation between pro-coagulant activity using TEG and expression of cell surface TF, so surface TF expression was evaluated here using flow cytometry with anti-CD142 antibody.
   - This task was completed in the previous grant year and covered in the Year 1 annual report (April 2019). We have determined TF expression on >20 different MSCs from different sources and identified MSCs with low, medium and high TF levels. Recently, we have noted that the same cells under different conditions vary in TF expression, demonstrating the importance of consistent handling and culture conditions. This type of testing will still be done on future cell preparations to evaluate them before animal administration.

C. Generate MSCs with TF "knocked down" (TF-KD cells) using shRNA/siRNA specific for human TF. Determine level of TF in TF-KD cells relative to controls treated similarly with a non-specific siRNA/shRNA using western blotting or flow cytometry.
   - Although we described progress on this task in the previous year's annual report, we are still working on an alternative method to reduce TF expression in MSCs.
   - We have had some difficulty getting high enough transfection efficiencies with human MSCs, except when using transient TF-specific siRNAs. Although the TF-specific siRNAs did reduce TF levels significantly (when evaluated by qRT-PCR), it has been difficult to obtain enough transient TF-knockdown (TF-KD) cells to perform many functional tests on the cells. Because these cells are primary (non-immortal) cells, they possess a limited proliferation potential, and in fact become less functionally active after a large number of population doublings. For this reason, we routinely use our cells for up to only approximately 20 population doublings. For our transfected TF-KD cells, we do get a decent number of transiently transfected cells (10-20% of the population as judged by GFP fluorescence following transfection with our vectors which also contain the GFP fluorescent marker). The number of cells is only enough to assay the TF mRNA level by qRT-PCR but not to do other functional testing. Additionally, since only 10-20% of the cells are positive for vector uptake, 80-90% are not. This level may not be enough to see a decrease in functional activity. Therefore, we tried to select stably transfected cells using puromycin resistance (also contained within the plasmid vector). Since only cells retaining the

plasmid vector can survive and grow in puromycin, the resulting population after selection will all contain the TF-KD plasmid. However, only a very small percentage of the cells that originally took up the plasmid transiently retained it stably after selection. Therefore we were unable to obtain a large number of stable TF-KD cells, and the cells we did obtain had undergone a large number of population doublings, causing poor growth and function probably due to an increased number of senescent cells in the expanded population. Thus we felt this method was unsuitable to use for determining what effects TF expression levels have on MSC function. Although we tried several different transfection methods to increase transfection efficiency (including lipofectamine, calcium phosphate, Viromer Red), none increased the transfection efficiency to a desirable level.

- Therefore, we adopted an alternate strategy. Over the course of this past year, we have adopted a retroviral infection method to reduce TF expression in MSCs with a high level of cell surface TF expression. The plasmid vector that we utilized for the TF-KD is also suitable for retroviral expression. Using this method, we have achieved a much higher efficiency for uptake and retention of the TF-KD virus and negative control virus (not specific for TF or any other gene). The virus produced also contains the gene encoding puromycin resistance but the gene encoding GFP is not included in the packaged virus. This allows us to use puromycin resistance to select a population of cells all containing stable copies of the shRNA. We have obtained and cryopreserved reasonable numbers of TF-KD cells for testing but have not yet used them for functional testing.
- Preliminary results using this method were presented in abstract and poster form at the RegenMed SA meeting in San Antonio in February 2020 (Delavan et al, "Reducing Tissue Factor in Mesenchymal Stromal Cells to Improve Safety").
- 100% of this task is done; we have obtained and cryopreserved reasonable numbers of TF-KD and control cells.

D. Test TF-KD cells for pro-coagulant activity using TEG and CAT. Ask if reducing the amount of TF reduces pro-coagulant activity.
   - Unfortunately, our research has recently been halted due to the COVID-19 pandemic. We are unsure when we will be able to work in the laboratory again at this point. When we return, this task is not likely to be our highest priority, but we hope to be able to perform coagulation assays on the TF-KD cells within the next 6 months.

E. Test TF-KD cells for functional activity using several assays being routinely used in our laboratory. These assays include a modified MLR assay to assess immunomodulation activity, IDO enzyme activity induction under inflammatory conditions, ability to polarize macrophages. Other assays may also be performed with these populations at a later time. Ask if reducing TF reduces functional activity.
   - Unfortunately, our research has recently been halted, due to the COVID-19 pandemic. We are unsure when we will be able to work in the laboratory again at this point. When we return, this task is not likely to be our highest priority, but we hope to be able to perform functional assays on the TF-KD cells within the next 6 months.

F. Develop and/or implement assays in our laboratory to evaluate MSC function in macrophage polarization.

- As previously described in prior progress reports, this task is complete. Although we tested several macrophage polarization assays described in the literature, one has worked more consistently than the others. We have implemented a macrophage polarization assay involving the use of human THP-1 monocytes. In this assay, co-culture with MSCs derived from bone marrow, adipose or umbilical cord blunts the pro-inflammatory (M1) response to treatment with LPS (demonstrated by decreased TNFa secretion) and augments the anti-inflammatory (M2) response (demonstrated by increased IL-10 secretion). We will continue to utilize this assay routinely for evaluation of new MSCs or MSC preparations for animal infusion; we feel that immune modulation involving the innate immune system is an important component of MSC activity. Results from this part of the project were included in a manuscript currently in press (Christy et al, "Use of Multiple Potency Assays to Evaluate Human Mesenchymal Stromal Cells", in press in *Journal of Trauma and Acute Care Surgery*).

G. Develop and/or implement assays in our laboratory to evaluate MSC function in vascular permeability.

- To analyze the effects of MSCs on vascular permeability (important for reduction of endothelial dysfunction and to maintain or restore blood brain barrier integrity after injury), we have been developing and/or testing assays to assess endothelial monolayer permeability.
- As described in the last quarterly report (Dec 2019), after testing several methods to analyze the effects of MSCs on endothelial monolayer permeability, we have settled on the ECIS (Electric Cell-Substrate Impedence Sensing) method. We were able to obtain the appropriate equipment (ECIS Model Z from Applied Biophysics, already in our institute but not in current use by another group) for these studies. With this instrument, we can measure impedence in real time in wells of a 96 well plate.
- A member of our technical staff, Mr. Delevan, has spearheaded these studies and has had success in establishing this method in our laboratory. Some of his early results were presented in abstract and poster form at the Military Health System Research Symposium in August 2019 (Delevan et al, "Assay Development to Assess the Influence of Cell Therapy Agents on Blood Brain Barrier Endothelial Cell Function"). He has used both HUVECs (human umbilical vein endothelial cells) and human blood brain barrier endothelial cells for these experiments, testing multiple conditions (cell plating density, matrix-based substrates, timing and agents used to disrupt the endothelial monolayer) to determine optimal conditions for assay.
- Due to the recent shutdown of our institute due to the COVID-19 pandemic, I am unable to access the most recent data, but will summarize here.
    1. Although both form tight monolayers after several days in culture, the optimal plating density for HUVECs and BBB cells is different.
    2. The HUVEC monolayer is easier to disrupt with treatment with agents such as TNFa. The BBB monolayer shows a smaller difference in the area under the curve with TNFa or other disruption agent (compared with vehicle control).

3. Conditioned medium from MSCs can affect the disruption and recovery of the HUVEC monolayer.
4. More concentrated conditioned medium appears to work more efficiently, but we have not yet optimized the conditions (when to apply the CM, how much, etc).

- Unfortunately, the ECIS Model Z instrument has needed repairs on two separate occasions, delaying the completion of this task. First, the circuit board needed to be replaced and the pin board updated. This repair necessitated shipping the equipment to the manufacturer. Following the return of the instrument to us, it was working well until it had a second problem. This time there was an issue with one of the circuit boards in the primary controller unit. The instrument was shipped back to the manufacturer a second time, but is now in good working order. We expect to be able to continue optimizing this assay in the future, but things are currently on hold until we can return to the laboratory. This assay may not be the highest priority once we are able to resume our research, but we do feel that it is a relevant and important assay for MSCs to be used in TBI. On the other hand, because this assay requires specialized equipment, it is less likely to translate into a routine assay that can be utilized by most laboratories.

H. At the end of testing, identify "good" and "bad" MSCs for in vivo testing. "Good" MSCs will be defined as MSCs with low TF and pro-coagulant activity and high potency in *in vitro* functional assays. In vitro potency will need to be validated *in viv*o (in Aim 2) to determine efficacy in vivo. (Note that we use "potency" to describe in vitro function and "efficacy" to describe function in vivo).

- We have already identified "good" and "bad" MSCs to be used for *in vivo* testing, based on their TF levels and performance in several functional assays (MLR, IDO, macrophage polarization). This task is completed.

Aim 2. Animal testing. In this aim we will determine dose response for "good" and "bad" MSCs in uninjured and injured animals (rats).

A. Generate animal protocol for rat TBI. Obtain IACUC approval.

- The initial rat study concerning injection of human MSCs into uninjured rats was carried out under an approved modification to Dr. Wu's animal protocol.
- The animal protocol for the model development phase of this study (by Dr. Darlingtonn) was approved, but an additional protocol will be developed for the full study.
- Although progress may be delayed due to the current ISR shutdown, we hope to obtain approval by the end of summer 2020.

B. Dose response study to determine maximum tolerated dose for MSCs injected intravenously into normal rats.

- Under a modification to Dr. Wu's existing animal protocol, the maximum tolerated dose for adipose (high TF) and bone marrow (low TF) MSCs has been determined. As predicted, the rats can tolerate more of the bone marrow MSCs with lower TF (up to 20 million/kg body weight for BM-MSCs; 10 million/kg for AD-MSCs).

- Testing with human MSCs in uninjured rats was carried out in this quarter, under an approved modification to Dr. Wu's IACUC protocol. Under this protocol, Dr. Wu previously showed that rat bone marrow-derived (low surface TF expression) MSCs are better tolerated than rat adipose-derived (high surface TF expression) MSCs, when administered intravenously via the tail vein. We anticipated a similar trend for the human MSCs, although we realized that the immune response in rats generated by human (xenogenic) cells could be even more pronounced. The MSCs were labeled with CMFDA/CFSE before administration to allow short-term biodistribution analysis. As shown below in Figure 1, the CFSE labeling procedure yielded efficient labeling of the cells (95-100% positive for all cell preparations).

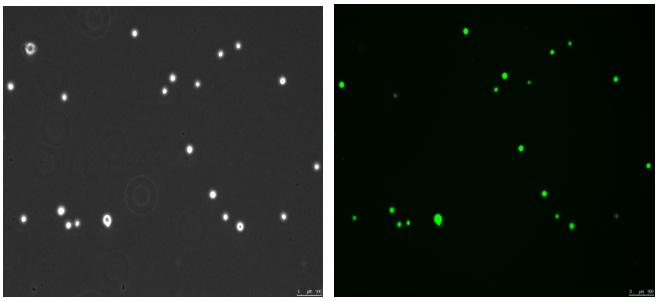


Figure 1. Human MSCs are very efficiently labeled with CFSE prior to injection. Left side, a bright field image of BM-MSCs prior to injection (in solution, after labeling and harvest). Right side, a fluorescent image of the same field showing CFSE+ cells. Although BM-MSCs are shown here, >95% labeling was seen with AD-MSCs as well.

- After labeling and cell harvest but prior to injection, an aliquot of cells was analyzed by flow cytometry to determine the percentage of cells labeled with CFSE and the percentage of cells positive for TF expression. Although each of the cell types had been tested for TF expression previously, the levels vary somewhat depending on conditions and we thought it was important to know the levels for each preparation at the time of injection.

4 rats each were injected with one of 4 cell types:

- BM-13 (bone marrow-derived, low TF <10%+)
- BM-55 (bone marrow-derived, moderate TF ~30%+)
- AD-88 (adipose-derived, high TF >70%+)
- AD-88 Treated (adipose-derived, moderate TF ~30%+)

- All cells were obtained commercially and used at low passage number (p.4-5 corresponding to approximately 12-18 population doublings). Cells were labeled with CFSE prior to detachment from tissue culture plastic, washed to remove excess label and culture medium, harvested with TrypLE, washed extensively to remove medium and

TrypLE, and cell number and viability was determined. The final pellet was suspended in PlasmaLyte at the appropriate cell number to obtain a dose of 10 million cells/kg of body weight (depending on the weight of each rat). Cells were injected intravenously via the tail vein. Blood was obtained at 1 hour before injection (baseline) and at 1 hr and 3 hr after MSC injection. Blood was analyzed by flow cytometry to determine the number of CFSE+ cells in the bloodstream at the different time points. In addition, immune cell distribution was analyzed at each of the time points to determine the effects of MSC injection on immune cell types and to determine if there are differences in response to BM-MSCs vs. AD-MSCs and whether differences in TF expression effect the response. Whole blood was analyzed for parameters related to clotting function. At sacrifice, several tissues were harvested and cell sections were prepared (including lung, liver, spleen) and are waiting to be stained with a human-specific anti-mitochondria antibody (Millipore Sigma, MAB1273) to detect any human cells that have infiltrated these tissues.

- Although our analysis is not yet complete, there are definite acute effects on both blood clotting parameters and immune cell distribution following IV injection of human MSCs. Similar to our previous findings with rat MSCs, human BM-MSCs and AD-MSCs are safe for injection into rats at the dose chosen (10M/kg). As shown in Figure 2, prothrombin time is increased following MSC administration at both time points.

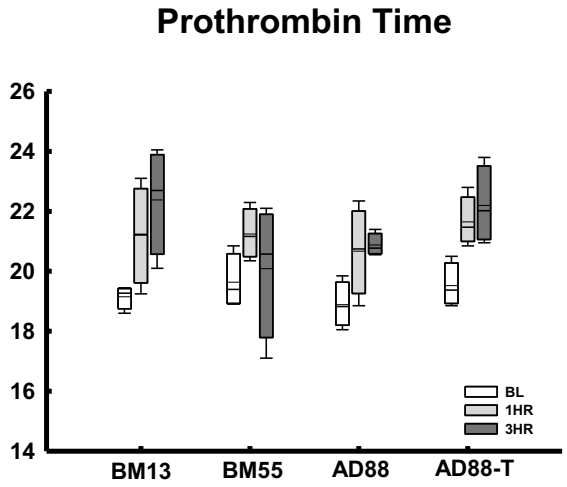


Figure 2. Prothrombin time increases for rat plasma following IV administration of human MSCs. BL, Baseline, 1 hr prior to MSC injection. 1 hr and 3 hr; 1 hr and 3 hr after MSC injection.

- As shown in Figure 3, platelet counts also change following MSC infusion, showing a decrease at both 1 hr and 3 hr after treatment. This suggests that the platelets may be interacting with the MSCs, potentially to facilitate their removal. These results suggest that an acute coagulation response was triggered by the injection of human MSCs.

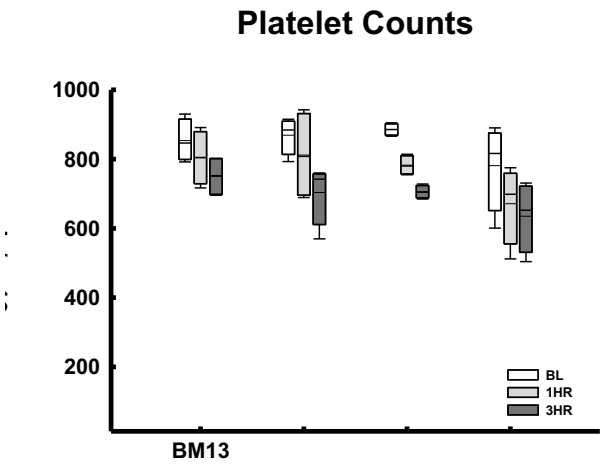


Figure 3. Human MSC administration decreases platelet counts in rat plasma following IV administration.

- When the immune cell distribution was analyzed by flow cytometry in rat blood at baseline and at 1 hr and 3 hr following human MSC administration, we observed several dramatic changes. First, we see a large increase in monocytes at both 1 hr and 3hr following infusion. At the same time, NK cells, B cells and neutrophils are all decreasing. These changes are not seen with injection of vehicle alone, but are similar to changes seen acutely following polytrauma. Further analysis is underway at this time. Clearly, the infusion of human MSCs elicits a strong acute response in the animals.

C. Biodistribution studies using labeled MSCs to determine how long they last in the bloodstream and where they go after administration.
   - Blood samples were taken at 1 hr and 3 hr post-MSC infusion and analyzed for the presence of labeled MSCs in the bloodstream by flow cytometry. Very few (<0.5% of total cells in the blood) labeled MSCs were detected in the blood at either time point.
   - Tissue samples were also taken from the lung, liver, spleen, brain of animals infused with CFSE-labeled human MSCs. Sections were fixed and analyzed for the presence of human MSCs using both the CFSE-fluorescence and an anti-human mitochondrial protein antibody. Similar to previous work described in the literature, MSCs were found in the lungs. Except for the lungs, human cells were undetectable in other tissues.

D. Implement rat TBI model in our laboratory.

- Establishing a closed-skull rat model for moderate TBI in our facility. We are using a weight-drop method to generate the closed-skull brain injury. Our group is experienced using the weight drop method to generate bone injury in the context of polytrauma (Wu

et al, *Am J Physiol Regul Integr Comp Physiol* 310:R323-R329, 2016). Although an open-skull brain injury is more commonly used in the literature for blunt impact TBI (skull is exposed surgically before the impact), we chose to use a closed-skull method because we felt that it was more relevant to military TBI injuries.

- As of the end of this year, 17 animals have been tested. At this point, we are close to defining the conditions to generate the appropriate level of brain injury for our testing. Thus far, we have tested several injury doses selected based on a survey of the literature. If the injury is generated using 455 gm weight at a height of 42 inches, all of the animals die within the first 30 minutes. If the weight is lowered to 390 gm dropped from a height of either 42 inches or 36 inches, all of the animals still die within 30 minutes. However, when a weight of 390 gm is dropped from 30 inches, the animals survive for at least 4 hr. Because we want the animals to survive for a longer period of time, we have allowed animals to recover to 24 hr at this point. From these animals, we have harvested blood at baseline, 30 min, 2 hr and 4 hr post-injury. The model used is shown in Figure 4. Preliminary analysis of several blood parameters was carried out as shown below in Figure 4. Prothrombin time is not significantly changed at 2 hr or 4 hr, but at this time only a small number of animals have been analyzed. Lactate and creatinine levels do change over the 4 hour period. Plasma prepared from blood taken at baseline and 4 hr post-injury was cryopreserved for further analysis. Thus far, we have analyzed S100B levels in the plasma by ELISA, which does not change significantly in the time frame analyzed (data not shown).
- At the time of death or sacrifice, brains were harvested and sections were prepared for immunohistochemistry. Tissue and plasma were also harvested from Sham animals subjected to all procedures except for the TBI. Although the focal region of injury is often very obvious in open-skull impact TBI models and can be used to estimate the degree of injury, we found that the region of injury is diffuse in our closed-skull model and cannot be easily measured.

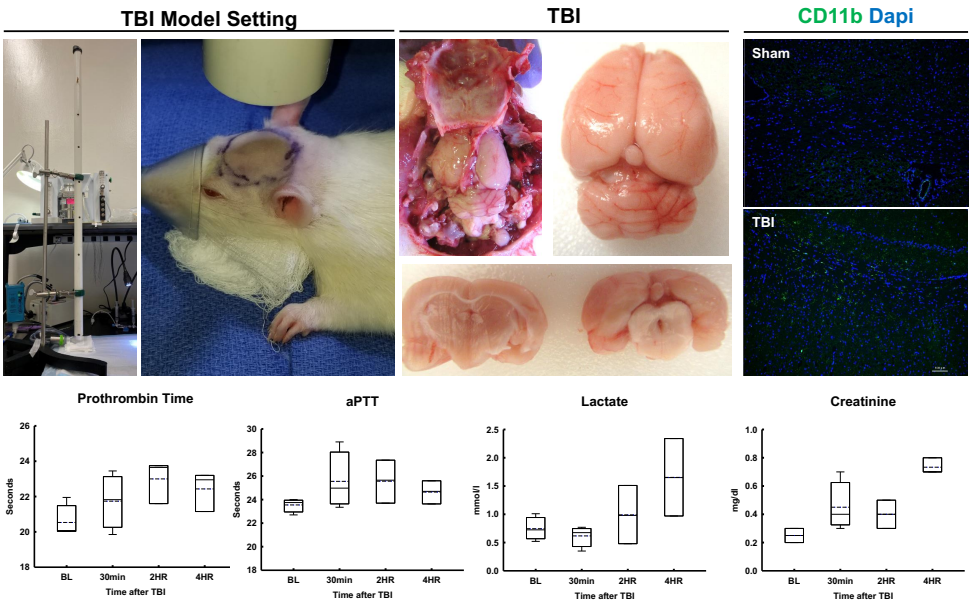


Figure 4. Closed-skull TBI model used in our laboratory. Top left, weight-drop apparatus. Second from left, a rat in position for weight drop. Skull is shaved and the targeted area is marked. Second from right, brain and brain sections after TBI. Right, sham and TBI brain sections stained for CD11b, which identifies macrophages and microglia, which increase post-TBI.

- Staining and analysis of brain and tissue sections has been carried out using immunohistochemistry. Antibodies used include those specific for CD11b (to mark macrophages & microglia), Neu N (to identify live neurons), MPO (to detect neutrophil infiltration), Iba 1 (to detect microglia/macrophages), OSP (to identify oligodendrocytes) and GFAP (to identify activated astrocytes). Fluoro Jade B is also used to identify degenerating neurons.
- Some representative results of immunohistochemistry are shown above in Figure 4 (CD11b) and below in Figure 5 (GFAP and OSP). Even at this short time period after the injury, some of the responses are quite obvious. Staining for CD11b, GFAP and Iba1 increase in intensity after injury, while NeuN and OSP decrease.

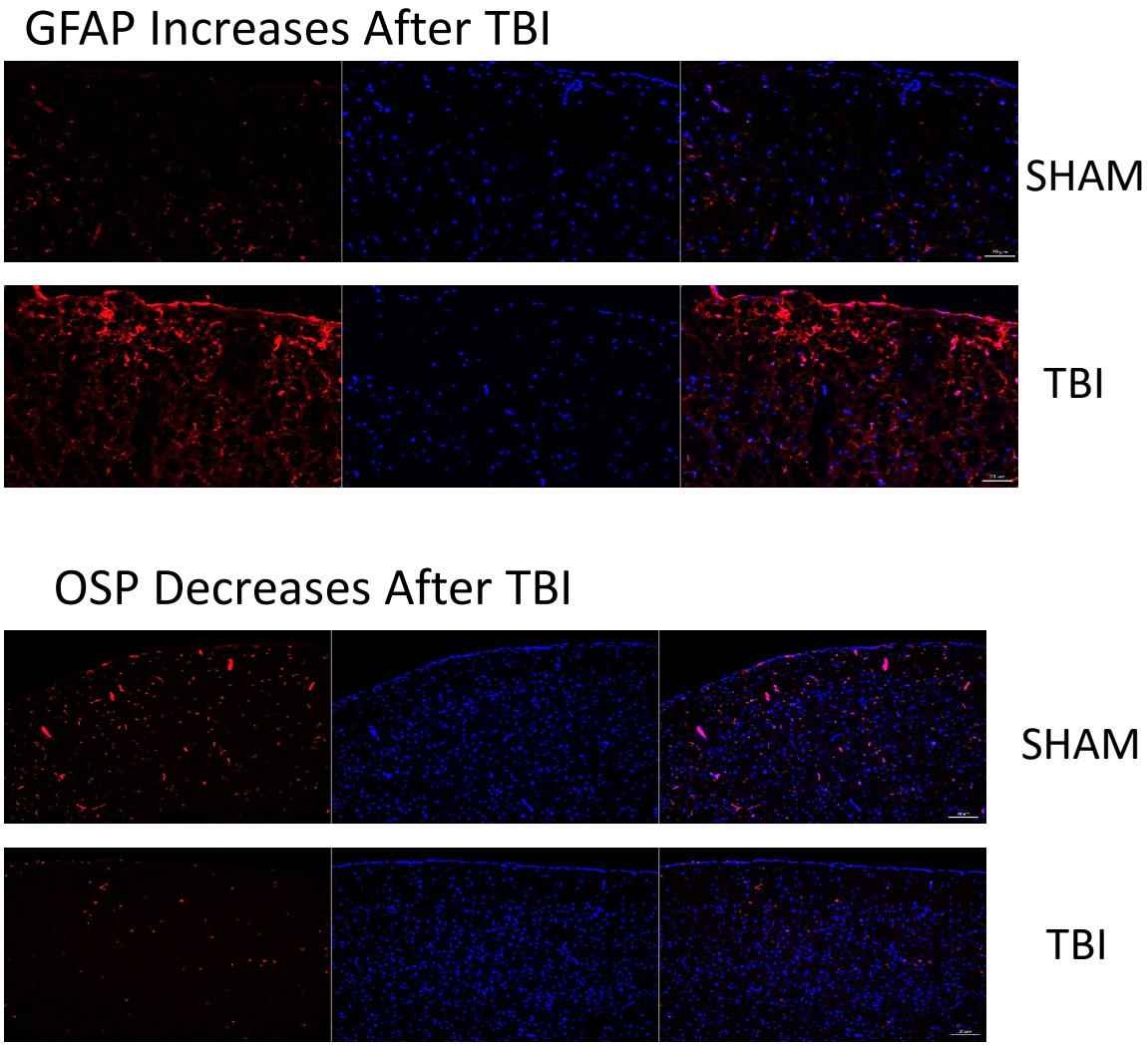


Figure 5. Brain sections taken from Sham rats and TBI rats taken at 4 hr. following injury. Sham rats were treated identically to TBI rats with the exception of the actual injury; their heads were shaved and they were anesthetized and placed in the weight-drop apparatus. Top; sections from one rat in each group (sham or TBI) were stained for GFAP (identifies activated astrocytes). Bottom; sections from one rat in each group (sham or TBI) were stained for OSP (identifies oligodendrocytes). Red color, antibody of interest (GFAP or OSP, left panels). Blue color, DAPI to identify nuclei (center panels). Right panels show an overlay of the two stains.

E. Dose response study to determine maximum tolerated dose for MSCs injected intravenously into rats subjected to TBI.
   - These studies have not been completed; they were slated to start at the end of the project year but had to be canceled due to the ISR shutdown due to the pandemic. Rats that were already resident at the ISR for this and other projects were transferred to a training protocol; new animals of the proper weight and age will be obtained when animal work resumes.The studies will be rescheduled for a later time after the resumption of research at the ISR.

F. **Aim 3. Administration of MSCs to rats subjected to TBI and analysis**.
   - Studies not yet begun, proposed for the 3rd year.

Thus far, two abstracts were generated from the animal work described in this part of the project:

- Wu X, Darlington DN, Christy BA, Liu B, Keesee JD, Cantu-Garza C, Garciamarcano J, Cap AP. Systemic Administration of Bone Marrow-Derived Mesenchymal Stromal Cells in Rats with Acute Traumatic Coagulopathy. Poster presentation, AABB Annual Meeting, San Antonio, TX, Oct. 2019.
- Wu X, Christy BA Herzig MC,Salgado C, Liu B, Keesee JD, Cantu-Garza C, Garciamarcano J, Darlington DN, Cap AP, Bynum J. Intravenous Administration of Human Mesenchymal Stem Cells Induces Coagulopathy Independent of Tissue Factor Expression. Submitted for presentation, AABB Annual Meeting in Baltimore, MD, Oct. 2020.

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.

Numerous training activities are available to our personnel, and many types of training are in fact required yearly for both civilian government personnel and contractors. In addition, our cell therapy team has recently received specialized training in equipment use, including additional training for normalization of data generated by the Agilent Seahorse and several types of imaging and statistical software.
In addition, the PI and many of our team attended and presented at several conferences in the past year, including RegenMed SA, AABB Annual Meeting, Military Health System Research Symposium (MHSRS), and the International Society for Cell Therapy Annual North American Regional meeting.
Our cell therapy team participates in twice-monthly meetings to discuss data and experimental strategy. This meeting is regularly attended by all of the participants in this project and other interested parties.

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.

Several abstracts, oral presentations and posters have been presented at local, regional and national meetings.

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 continue and finish the remaining *in vitro* studies described in Aim 1. Once TBI model development is complete, the dose response in injured and uninjured animals will be compared (it is very possible that the MSC tolerance in injured animals will be altered). Administration of MSCs with different characteristics to rats subjected to TBI will be carried out as soon as possible after the resumption of animal work at the ISR.

4. **IMPACT:** Describe 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 results from this study have not yet been published. We do anticipate making significant contributions to both the cell therapy and TBI fields.

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.

Nothing to Report.

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:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

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.

During this reporting period, we have had several delays to our research plan. First, for the vascular permeability experiments described in Aim 1, we have tested several methods and settled on a method involving ECIS (Electric Cell-Substrate Impedance Sensing). This method involves specialized equipment that was already present at the ISR but had not been in use for quite a while. Therefore we have had to have major maintenance done, involving sending the equipment back to the manufacturer twice and resulting in some long delays. We currently do have the assay working and anticipate being able to complete this part of the study within the next year. A more serious delay is currently occurring due to the shutdown of all research activities (including animal work) at the ISR due to the Covid-19 pandemic. This has delayed all of our activity and we do not yet know how long it will last. Following resumption of our research, the animal studies will be further delayed because new rats will need to be obtained and our animal rooms were repurposed for other uses and will need to be restored. As soon as research at the ISR is resumed, we will continue with this work.

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. No human subjects to be used.

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

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).*

Published Manuscript:

1. Christy BA, Herzig MC, Delavan C, Cantu C, Salgado C, Bynum JA & Cap AP. Human Primary Fibroblasts Perform Similarly to MSCs in Assays Used to Evaluate MSC Safety and Potency. *Transfusion* 2019, 59(S2):1593-1600.

Accepted Manuscripts:

2. Christy BA, Herzig MC, Delavan C, Salgado C, Cantu C, Lovelace S, Jensen K, Garcia L, Montgomery RK, Bynum JA & Cap AP. Use of Multiple Potency Assays to Evaluate Human Mesenchymal Stromal Cells. In Press in *J. Trauma Acute Care Surg*., 2020.
3. Chance T, Herzig MC, Christy B, Delavan C, Rathbone C, Cap AP, Bynum J. Human Mesenchymal Stromal Cell Source and Culture Conditions Influence Extracellular Vesicle Angiogenic and Metabolic Effects on Human Endothelial Cells. In Press in *J. Trauma Acute Care Surg*, 2020.

Submitted Manuscripts:

4. Herzig MC, Christy BA, Montgomery RK, Delavan CP, Jensen KJ, Lovelace SE, Cantu C, Salgado CL, Cap AP, Bynum JA. Interactions of Human Mesenchymal Stromal Cells with Peripheral Blood Mononuclear Cells in a Mitogenic Proliferation Assay. Submitted to *J. Immun. Methods*, 2020.
5. Herzig MC, Delavan C, Jensen KJ, Cantu C, Montgomery RK, Christy BA, Cap AP, Bynum JA. A Streamlined Lymphocyte Proliferation Assay Using Mixed Lymphocytes for Evaluation of Human Mesenchymal Stem Cell Immunomodulation Activity. Submitted to *J. Immun. Methods*, 2020.

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).*

Published Abstracts:

1. Christy BA, Herzig MC, Delavan C, Kirian R, Olsen T, Ahsan T, Cap AP, Rowley J, Bynum JA. Mesenchymal Stem Cells Grown in a Bioreactor are Functionally Similar to Those Grown in Monolayer Culture. Oral presentation, AABB Annual Meeting, San Antonio, TX, Oct. 2019.
2. Christy BA, Herzig MC, Srinivasan A, Delavan C, Cap BM, Bynum JA, Cap AP. Refrigerated Mesenchymal Stem Cells Maintain Viability and Function for At Least 8 Days. Poster presentation, AABB Annual Meeting, San Antonio, TX, Oct. 2019.
3. Wu X, Darlington DN, Christy BA, Liu B, Keesee JD, Cantu-Garza C, Garciamarcano J, Cap AP. Systemic Administration of Bone Marrow-Derived Mesenchymal Stromal Cells in Rats with Acute Traumatic Coagulopathy. Poster presentation, AABB Annual Meeting, San Antonio, TX, Oct. 2019.
4. Herzig MC, Cantu-Garza C, Kamucheka R, Delavan CP, Christy BA, Bynum JA, Cap AP. A Rapid Mixed-Lymphocyte Response Assay for Immunomodulation Based on Inhibition of Apoptosis. Poster presentation, AABB Annual Meeting, San Antonio, TX, Oct. 2019.
5. Christy BA, Herzig MC, Delavan C, Kirian R, Olsen T, Ahsan T, Cap AP, Rowley J, Bynum JA. Mesenchymal Stem Cells Grown in a Bioreactor are Functionally Similar to Those Grown in Monolayer Culture. Poster presentation, International Society for Cell and Gene Therapy (ISCT) North American regional meeting, Madison, WI, Sept. 2019.
6. Christy BA, Herzig MC, Srinivasan A, Delavan C, Cap BM, Bynum JA, Cap AP. Refrigerated Mesenchymal Stem Cells Maintain Viability and Function for At Least 8 Days. Poster presentation, International Society for Cell and Gene Therapy (ISCT) North American regional meeting, Madison, WI, Sept. 2019.

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.*

Other abstracts in the last year not listed above:

1. Herzig MC, Cantu C, Montgomery RK, Kamucheka RM, Delavan CP, Christy BA, Bynum JA, Cap AP. A Rapid Mixed Lymphocyte Response Assay for Immunomodulation Based on Inhibition of Apoptosis. Oral presentation, Military Health System Research Symposium, Kissimmee, FL, Aug. 2019.
2. Christy BA, Cantu C, Parida BK, Herzig MC, Bynum JA, Cap AP. Human Mesenchymal Stromal Cell Interactions with Innate Lymphoid Cells. Oral presentation, Military Health System Research Symposium, Kissimmee, FL, Aug. 2019.
3. Delavan C, Christy B, Becerra S, Herzig M, Bynum J, Cap AP. Assay Development to Assess the Influence of Cell Therapy Agents on Blood Brain Barrier Endothelial Cell Function. Poster presentation, Military Health System Research Symposium, Kissimmee, FL, Aug. 2019.
4. Herzig, MC; Cantu, C; Montgomery, RK; Delavan, CP; Christy, BA; Bynum, JA; Cap, AP. A Rapid Mixed Lymphocyte Response Assay for Immunomodulation. Poster presentation, Military Health System Research Symposium, Kissimmee, FL, Aug. 2019.
5. Herzig MC, Christy BA, Cantu C, Delavan C, Bynum JA, Cap AP. Human Mesenchymal Stromal Cells have a Therapeutic Window for Immunosuppression in the Mixed Lymphocyte Reaction Assay. Poster presentation, Military Health System Research Symposium, Kissimmee, FL, Aug. 2019.
6. Wu X, Christy B, Herzig MC, Salgado C, Liu B, Keesee JD, Cantu-Garza C Garciamarcano J, Darlington DN, Cap AP, Bynum J. Intravenous Administration of Human Mesenchymal Stem Cells Induces Coagulopathy Independent of Tissue Factor Expression. Submitted for oral or poster presentation at AABB Annual Meeting, Baltimore, MD, Oct. 2020.

- **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.

Not applicable.

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

Nothing to Report yet.

- **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".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Barbara A. Christy, PhD
Project Role: Principal Investigator
Researcher Identifier: 0000-0001-5318-653X
Nearest person month worked: 6
Contribution to Project: Oversaw entire project, designed & carried out experiments, performed administrative tasks.

Name: Maryanne C. Herzig, PhD
Project Role: Co-Investigator
Nearest person month worked: 4
Contribution to Project: Helped design & carry out all experiments.

Name: James A. Bynum, PhD
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to Project: Intellectual contributions & administrative support.

Name: Xiaowu Wu, MD
Project Role: Co-Investigator
Nearest person month worked: 6
Contribution to Project: Designed & performed animal experiments.

Name: Daniel Darlington, PhD
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to Project: Design of animal experiments; wrote animal protocol.

Name:	Andrew P. Cap, MD, PhD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to Project:	Provided intellectual contributions and administrative support.

Name:	Christopher Delavan, MS
Project Role:	Research Technician
Nearest person month worked:	6
Contribution to Project:	Carried out much of the work described in this project, including MSC culture, assay development and more.

Name:	Isaac Abaasah, MS
Project Role:	Research Technician
Nearest person month worked:	4
Contribution to Project:	Provided technical support.

Name:	Christi Salgado, MS
Project Role:	Research Technician
Nearest person month worked:	4
Contribution to Project:	Provided technical support, animal support.

Name:	Gema Barrera, MS
Project Role:	Research Technician
Nearest person month worked:	1
Contribution to Project:	Flow cytometry analysis and interpretation.

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 in senior personnel.

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.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Dr. Lora Watts, University of the Incarnate Word, San Antonio, TX.
Dr. Watts is a collaborator who has provided consultation during this grant period, to advise us in setting up our rat model for TBI.

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.
See attached file.

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.

Mesenchymal Stem Cell Therapy for Traumatic Brain Injury

Award #BA170080

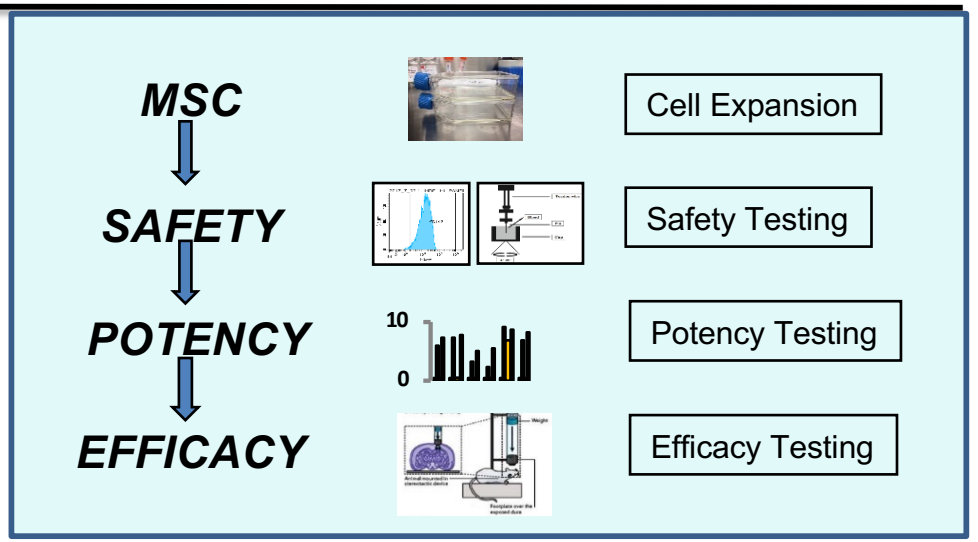
PI: Barbara Christy, PhD **Org:** US Army Institute of Surgical Research **Award Amount:** $1,455,907

Study Aims

- Objective 1: Develop *in vitro* testing protocols to evaluate safety and efficacy of MSCs.
- Objective 2: Preclinical testing for the identification of safe MSC dose in rats.
- Objective 3: Preclinical efficacy testing in a rat model of TBI.

Approach

- Multiple human MSC products will be evaluated for pro-coagulant potential to predict safety for IV delivery.
- Assays to evaluate potency (including immunomodulation, endothelial permeability) will be developed and implemented.
- Maximum tolerated dose will be determined in injured and non-injured rats; distribution & retention of cells will be followed.
- MSCs will be infused into rats subjected to TBI; effects on brain damage & recovery will be monitored.



Characterization of safety & potency underway for multiple MSCs.
Development of macrophage polarization and blood-brain barrier endothelial cell permeability are ongoing (potency testing).

Timeline and Cost

Activities / CY	18	19	20	
In Vitro Safety Testing				
In Vitro Potency Testing				
MSC dosing/distribution (rats)				
MSC testing in TBI				
Estimated Budget ($K)	**$454**	**$540**	**$462**	

Updated: April 17, 2020

Goals/Milestones (Example)

CY18 Goals –In vitro testing to identify "good" & "bad" MSCs

- In vitro safety testing
- In vitro potency testing (including new assay development)

CY19 Goal – Preclinical dosing and distribution studies

- Establish TBI protocols in our laboratory
- Determine maximal tolerated dose in both injured and uninjured rats
- Label cells and follow distribution & survival in injured and uninjured rats

CY20 Goal –

- Infuse "good" and "bad" MSCs into rats subjected to TBI
- Evaluate brain injury and recovery using multiple methods

Comments/Challenges/Issues/Concerns

- No major issues at this time

Budget Expenditure to Date

Projected Expenditure: $994K
Actual Expenditure: $995K