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14. ABSTRACT

Traumatic brain injury (TBI) is a significant cause of death/disability in military & civilian populations. Cell therapy with mesenchymal stem cells (MSCs) shows promise for TBI treatment. Not all MSCs are equivalent, thus 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 & potency of cell therapy products prior to administration. MSCs with different assay profiles will be compared in preclinical testing. 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 therapy impact will be measured using a combination of histological, functional and behavioral testing. Long term consequences of TBI are devastating, so treatments reducing damage are of tremendous importance.

15. SUBJECT TERMS

Traumatic brain injury, cell therapy, mesenchymal stem/stromal cells

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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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, not completed yet).

Aim 3. Testing of MSC administration for TBI. This goal was slated for the 3rd year of the proposal, but was not completed due to the COVID-19 shutdown. Following the resumption of research activity, these studies did not receive priority over core-funded activities also delayed due to COVID. Our facility has only recently started to operate back at 100% capacity in July 2021 (0% completed).

What was accomplished under these goals?

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 and some of the results were published in Results from this part of the project were included in a manuscript (Christy et al, Use of Multiple Potency Assays to Evaluate Human Mesenchymal Stromal Cells, *J. Trauma Acute Care Surg*, 2020, 89(S2):S109-S117).

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. We have determined TF expression on >20 different MSCs from different sources and identified MSCs with low, medium and high TF levels (see examples in Figure 1 below). We have also 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.

MSC	Tissue of Origin	%CD142 +
BM1	Bone Marrow	30.3
BM2	Bone Marrow	28.2
BM3	Bone Marrow	11.4
BM4	Bone Marrow	18.2
BM5	Bone Marrow	11.1
BM6	Bone Marrow	9.5
AD1	Adipose	75.3
AD2	Adipose	83.8
AD3	Adipose	84.3
UC1	Umbilical Cord	72.1
UC2	Umbilical Cord	91.1
UC3	Umbilical Cord	90.4

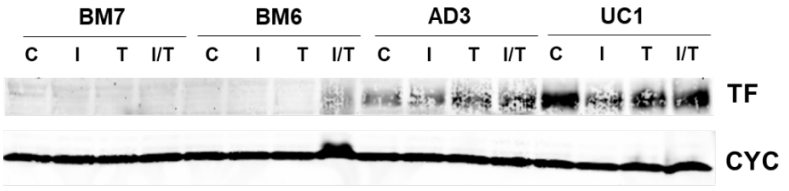


Figure 1. Human MSCs derived from umbilical cord and adipose tissue show a higher level of TF expression than bone marrow-derived MSCs. **Top.** Cell surface TF expression was determined by flow cytometry using anti-CD142 (TF) antibody. BM1-6; bone marrow-derived MSCs from 6 different donors. AD1-3; adipose-derived MSCs from 3 different donors. UC1-3; umbilical cord-derived MSCs from 3 different donors. **Bottom.** Western blot detection of tissue factor protein with anti-TF antibody (CD142). Representative BM-MSCs (BM6, BM7) with low TF expression are shown in comparison with representative AD-MSC (AD3) and UC-MSC (UC1) with higher TF.

- Rheometry was performed on a subset of MSCs mixed with human plasma (Figure 2). While all human MSCs tested showed pro-coagulant activity, the kinetics and strength of clot formation vary between different human MSCs and point out the need for evaluation prior to human administration.

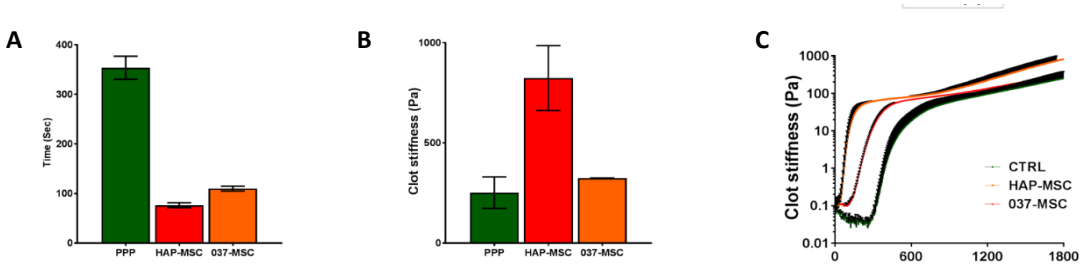


Figure 2. Evaluation of clotting time, clotting kinetics and clot stiffness by rheometry. One AD-MSC (labeled HAP-MSC) and one BM-MSC (labeled 037-MSC) were mixed with human platelet-poor plasma (PPP) at a concentration of $2x10^4$/ml and analyzed by rheometry. Each sample was analyzed in triplicate. A. R-time (seconds); time to clot initiation. B. Clot Stiffness. C. Kinetics of changes in clot stiffness (average of triplicates).

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.

- As described in previous reports, we have had some difficulty getting enough TF-KD cells for experimentation with our initial strategy.
- Although the TF-specific siRNAs did reduce TF levels significantly (when evaluated by qRT-PCR), it was difficult to obtain enough transient TF-knockdown (TF-KD) cells to perform functional testing. Because MSCs 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 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. Unfortunately, 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 using 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, research on this aim was delayed due to the COVID-19 pandemic. Although we do have cryopreserved TF-KD cells ready for testing, we were unable to carry out these experiments before the end of this project period. We do anticipate testing them at a future time to answer this important question.

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, research on this aim was delayed due to the COVID-19 pandemic. Although we do have cryopreserved TF-KD cells ready for testing, we were unable to carry out these experiments before the end of this project period. We do anticipate testing them at a future time to answer this important question.

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

- 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 TNFα 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. See figure 3 below for representative data from this assay.

- Results from this part of the project were included in a manuscript (Christy et al, Use of Multiple Potency Assays to Evaluate Human Mesenchymal Stromal Cells, *J. Trauma Acute Care Surg*, 2020, 89(S2):S109-S117).

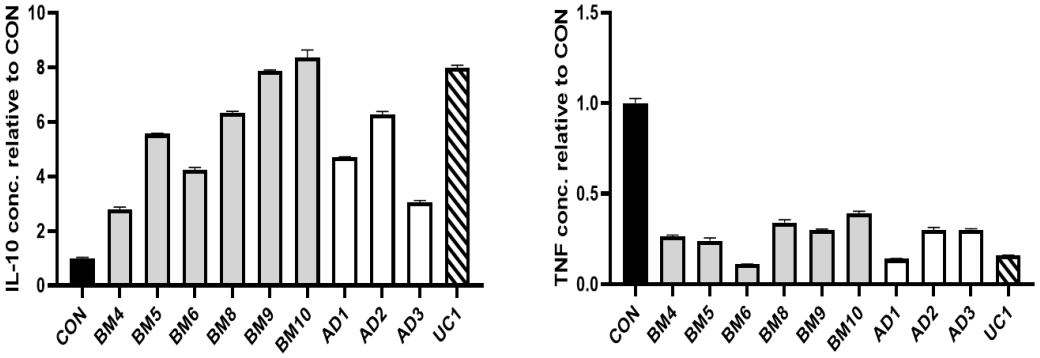


Figure 3. Individual MSCs differ in their ability to influence macrophage polarization. MSCs were co-cultured with THP-1 human monocytes in a ratio of 1:10 (MSC:THP1). Cultures were stimulated with LPS and conditioned medium was harvested after 6 hr and 24 hr of treatment. The concentration of IL-10 and TNF□□secreted by the THP1 cells into the medium was measured by ELISA. An increase in IL-10 and decrease in TNF□ reflects an increase in M2-like macrophages relative to M1-like macrophages. Left; increase in IL-10 concentration compared with control THP1 cells alone treated with LPS. Right; decrease in TNF□ concentration compared with THP1 cells alone treated with LPS. Values shown are from 6 hr after LPS treatment; 24 hr samples are not shown.

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.
- After testing several methods to analyze the effects of MSCs on endothelial monolayer permeability, we 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.
- These experiments are not completely finished, due to 3 factors causing delay:

- Due to the Covid-19 pandemic; our institute was completely shut down for a period of time.
- Re-opening of the institute has been slow. We started out at 25% occupancy of the building in summer 2020, moved to 50%, then back to 25% when a regional outbreak was occurring. We have only just fully re-opened for 100% occupancy of our building (in July 2021).
- 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.

Although this task has not been fully completed, we can summarize the conclusions we have reached so far:

- Although both form tight monolayers after several days in culture, the optimal plating density for HUVECs and BBB cells is different.
- 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).
- Conditioned medium from MSCs can affect the disruption and recovery of the HUVEC monolayer.
- 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).
- We do feel that the vascular permeability assay is a relevant and important assay for evaluation of MSCs to be used for TBI or other trauma. On the other hand, because this assay requires very 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 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).

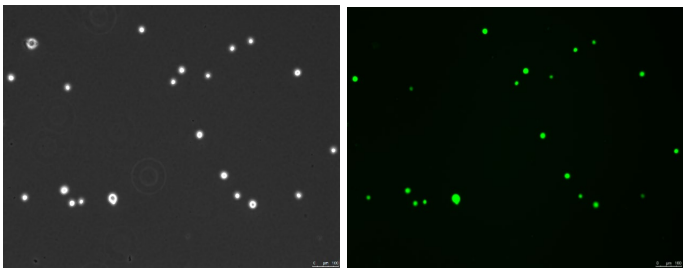
A. Generate animal protocol for rat TBI. Obtain IACUC approval.

- 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.
- Unfortunately, experimental progress was delayed/halted due to the Covid-19 pandemic. Animal experimentation at our institute was delayed even more than laboratory work, because the animal facility was initially closed and converted into potential human patient overflow space. It took quite a long time to get animal experimentation up and running, and mission-critical core-funded projects were the initial priority.

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 under the 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. 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).



Bright Field Image Fluorescent Image

- **Figure 4.** 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.
- Acute effects on both blood clotting parameters and immune cell distribution following IV injection of human MSCs were observed. 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 5 below, prothrombin time is increased following MSC administration at both time points.

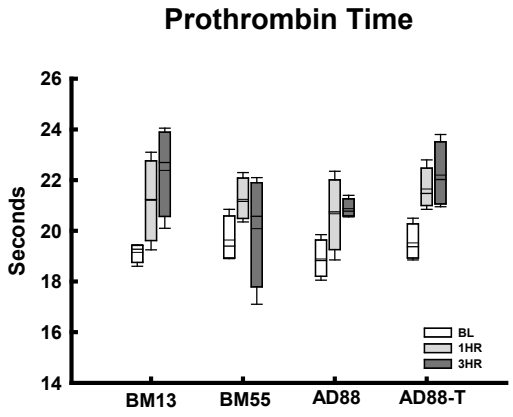


Figure 5. Prothrombin time increases for rat plasma following IV administration of human MSCs. BL, Baseline, 1 hr prior to MSC injection. 1 hr and 3 hr; 1 hr and 3 hr after MSC injection.

- As shown in Figure 6, 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.

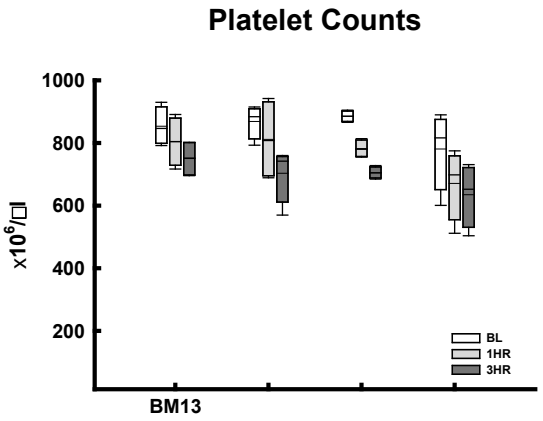


Figure 6. Human MSC administration decreases platelet counts in rat plasma following IV administration.

- 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 (Wuet 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 project, 17 animals have been tested. We are close to defining the conditions to generate the appropriate level of brain injury for our testing. 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 7. Preliminary analysis of several blood parameters was carried out as shown below in Figure 7. 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.

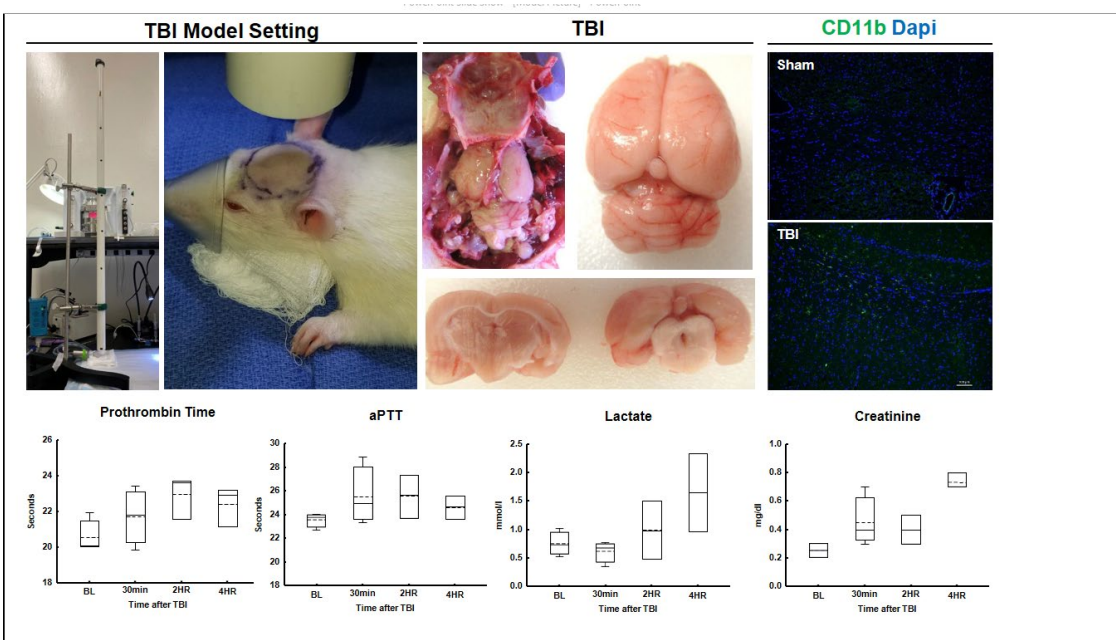


Figure 7. Closed-skull TBI model used in our laboratory. Top left, weight-drop apparatus. Second from left, a rat in position for weight drop. Skull is shaved and the targeted area is marked. Second from right, brain and brain sections after TBI. Right, sham and TBI brain sections stained for CD11b, 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 7 (CD11b) and below in Figure 8 (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.

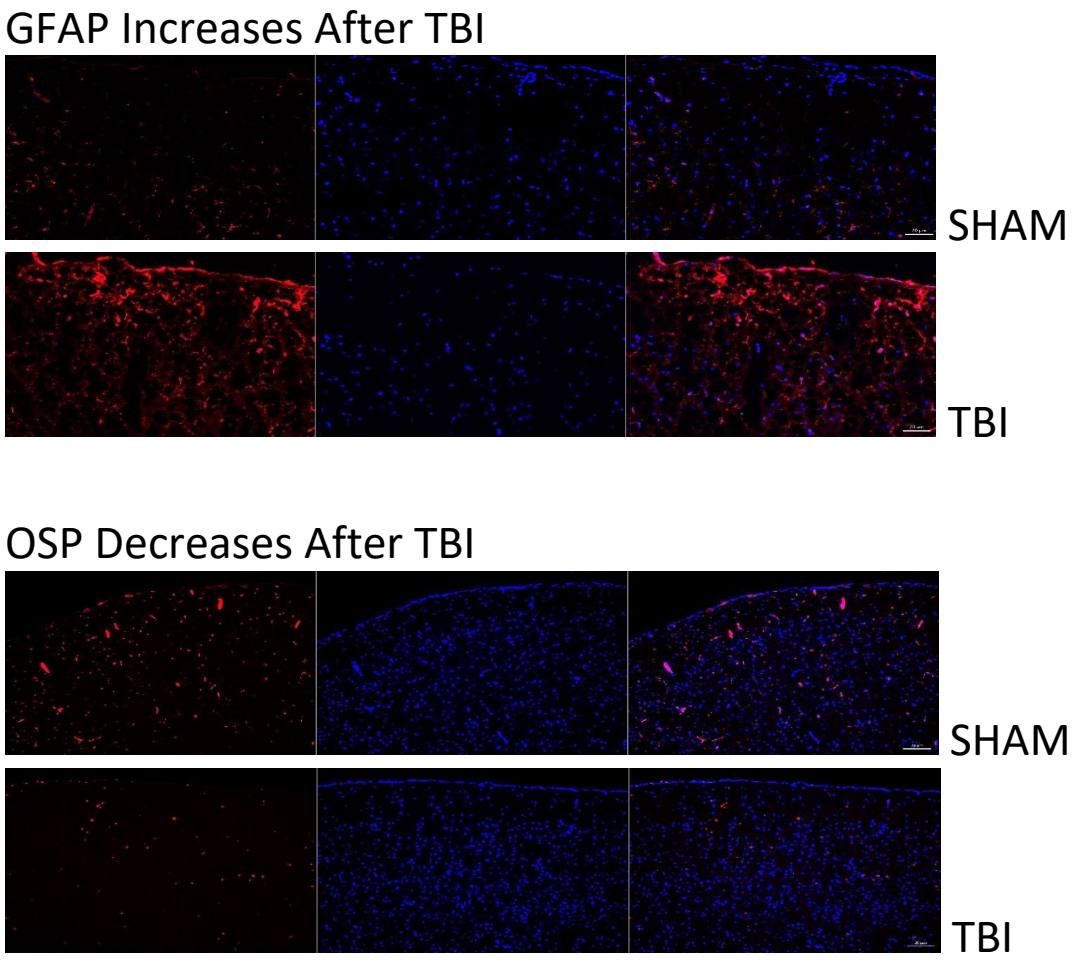


Figure 8. Brain sections taken from Sham rats and TBI rats taken at 4 hr. following injury. Sham rats were treated identically to TBI rats with the exception of the actual injury; their heads were shaved and they were anesthetized and placed in the weight-drop apparatus. Top; sections from one rat in 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 last 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.
- Although the ISR is now fully open (as of July 2021), animal projects have been significantly delayed. Priority is given to mission-critical core-funded projects.

Aim 3. Administration of MSCs to rats subjected to TBI and analysis.

- This part of the study was slated to begin at about the same time that the ISR had to shut down due to the Covid-19 pandemic.
- The ISR as a whole shut down completely in March 2020 and all of our personnel worked remotely at first.
- Laboratory personnel were allowed to come back and carry out some experiments at the end of summer 2020. We were only allowed to be at 25% building capacity, which meant that we could not accomplish as much. Allowed capacity was initially raised to 50%, but was decreased again to 25% when Covid cases began rising in winter 2020. We have only recently (July 2021) been able to operate at 100% full capacity.
- Our animal facilities completely shut down and all animals were either sacrificed, moved to other facilities, or transferred to training protocols. All equipment in the facility was moved out to repurpose the facility for human ICU overflow patient use (which was thankfully not needed). Animal work was completely shut down for many months. Once the human ICUs were dismantled, the animal facility took some time to get running again (partly due to the reduced staffing).
- Once animal studies began again, the mission-critical core-funded projects (also delayed due to Covid) took priority over everything else.

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

Investigators & some technical staff attended these conferences:

- Military Health System Research Symposium
- AABB (American Assn. of Blood Banks) Annual Meeting
- ISCT (International Society for Cell & Gene Therapy) Annual Meeting
- RegenMed SA Annual Meeting (Local, UT San Antonio, UT Health San Antonio, BioBridge Global & other entities)
- Military City USA Meeting (Local, with UT Health San Antonio)

How were the results disseminated to communities of interest?

PI and co-investigators regularly present oral presentations at meetings and joint seminars with the local scientific community, mostly at UT San Antonio and UT Health San Antonio.

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

Nothing to Report (final report).

4. IMPACT:

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

Although most commonly used rat models involve open-skull trauma, we have developed a closed-skull model that we believe is more relevant for combat-related trauma in the real world. Since one consequence of the closed-skull injury is that the brain lesion is more diffuse and harder to quantify, we are still working to extend our model with a smaller impact area by modifying the apparatus that we use to generate the injury.
Establishment of the closed-skull TBI model in our institution will also be valuable for polytrauma studies going forward.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to Report.

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

We encountered many problems causing delays in the work. The main disruption was due to the Covid-19 pandemic, which halted all work at our institute in March 2020. Our animal facility was completely shut down for many months (even when we were allowed to resume some laboratory work at a reduced level). All animals were transferred out of the facility. This was done in order to convert the animal facility for potential ICU patient use if hospitals became overwhelmed with Covid patients (which thankfully did not happen). Additionally, laboratory personnel were only allowed to work at a reduced level throughout the pandemic (from 25%-50% occupancy of the buildings). 100% capacity has only recently been allowed again (as of July 2021). When animal work began again, all projects were behind and priority was given to the most mission-critical core-funded projects. Another problem we encountered was that our money for the 3rd year of this project was FY19 dollars; thus we had to spend that money while things were shut down for the pandemic. Therefore, when animal work resumed we no longer had money to keep the project going after work was restarted. We do have reagents that were ordered at that time; we will continue to use these reagents to extend the model even though the project period has ended.

Changes that had a significant impact on expenditures

Nothing to report beyond what was noted previously.

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

Not applicable.

Significant changes in use or care of vertebrate animals

No changes from approved protocols.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS:

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

Journal publications.

- 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.
- 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 vitro*. *J. Trauma Acute Care Surg*, 2020, 89(S2):S100-S108.
- Christy BA, Herzig MC, Delavan C, Abaasah I, Cantu C, Salgado C, Lovelace S, Garcia L, Jensen K, Montgomery R, Cap AP, Bynum JA. Use of Multiple Potency Assays To Evaluate Human Mesenchymal Stromal Cells. *J. Trauma Acute Care Surg*, 2020, 89(S2):S109-S117.
- Lowry LE, Herzig MC, Christy BA, Schaefer R, Pati S, Cap AP, Bynum JA. Neglected No More: Cellular Therapies in Traumatic Injury. *Stem Cell Reviews and Reports*, DOI 10.1007/s12015-020-10086-7.
- 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. *J. Immunol. Methods*, 2021 Jan;488:112915. Doi:10.1016/j.jim.2020.112915. Epub 2020 Nov 16.
- 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. *J. Immunol. Methods* 2021 Feb17:113000.doi:10.1016/j.jim.2021,113000.
- Wu X, Darlington DN, Christy BA, Liu B, Keesee JD, Salgado CL, Bynum JA, Cap AP. Intravenous Administration of Mesenchymal Stromal Cells Leads to a Dose Dependent Coagulopathy as a Significant Limitation in the Treatment of Acute Trauma with Hemorrhagic Shock. Submitted, *J. Trauma Acute Care Surg.*, 2021.

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

None.

Other publications, conference papers and presentations.

Conference Abstracts

- 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.
- 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.
- Christy B, Herzig M, Wu X, Cap A, Bynum J. Cell Therapy for Traumatic Injury: Promise and Challenges. Oral presentation, RegenMed SA 2020, San Antonio, TX, Feb. 2020.
- Wu X, Christy B, Darlington DN, Salgado C, Liu B, Keesee JD, Cap AP, Bynum JA. Development of a rat model for sub-lethal traumatic brain injury with acute coagulopathy. Military Health System Research Symposium, August 2020 (virtual).
- *Wu X, Christy B, Herzig MC, Salgado C, Cantu-Garza C, Liu B, Keesee JD, Garciamarcano J, Darlington DN, Cap AP, Bynum J. Intravenous Administration of Human Mesenchymal Stromal Cells Induces Coagulopathy Independent of Tissue Factor Expression. Poster presentation, AABB Annual Meeting (virtual), October 2020.
- *Chance TC, Herzig MC, Christy BA, Delavan C, Rathbone CR, Cap AP, Bynum JA. Human Mesenchymal Stromal Cell Source and Culture Conditions Influence Extracellular Vesicle Angiogenic and Metabolic Effects on human Endothelial Cells. Poster presentation, AABB Annual Meeting (virtual), October 2020.

- **Website(s) or other Internet site(s)**

Not applicable.

- **Technologies or techniques**

Not applicable.

- **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: Barbara A. Christy, PhD
Project Role: Principal Investigator
Researcher Identifier: 0000-0001-5318-653X
Nearest person month worked: 6/year
Contribution to Project: Oversaw entire project, experimental design & performance, administrative tasks.

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

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

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

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

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

Name: Christopher Delavan, MS
Project Role: Research Technician
Nearest person month worked: 6/year
Contribution to Project: Carried out 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/year
Contribution to Project: Provided technical support.

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

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

Name: Tiffany Heard, MS
Project Role: Research Technician
Nearest person month worked: 2/year
Contribution to Project: Provided technical support.

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

No changes to senior/key personnel.

What other organizations were involved as partners?

Not applicable.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

See attached quad chart on next page.

Mesenchymal Stem Cell Therapy for Traumatic Brain Injury

Award #BA170080

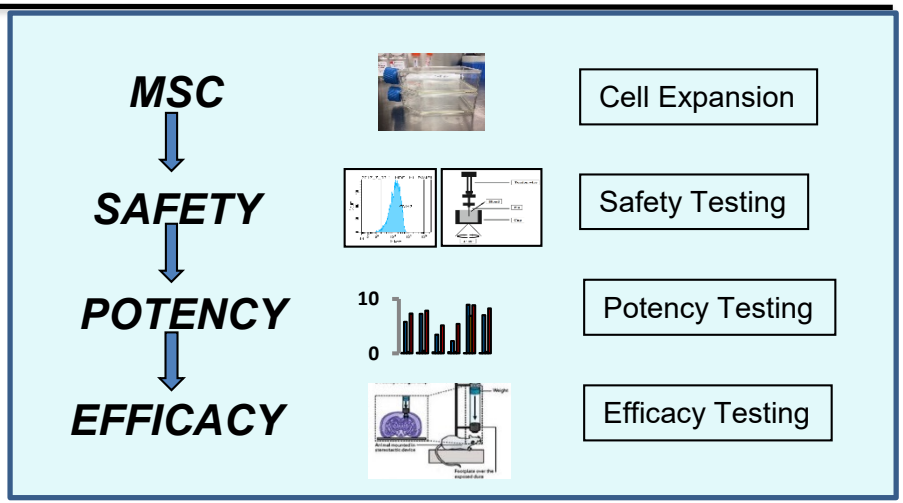
PI: Barbara Christy, PhD **Org:** US Army Institute of Surgical Research **Award Amount:** $1,455,907

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: July 14, 2021

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

- Covid-19 delays

Budget Expenditure: $1455.9K