AWARD NUMBER: W81XWH-17-1-0602, Log #SC160218

TITLE: Treatment of Spinal Cord Ischemia with Cell Impermeant-Based Resuscitation

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REPORT DATE: SEPTEMBER 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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Treatment of Spinal Cord Ischemia with Cell Impermeant-Based Resuscitation

The purpose of the work in the first year of the project was to determine the optimal size of PEG polymer that would be most effective in later trials in spinal cord injury models. Based on known mechanisms of injury and action of PEG-20k in the peripheral circulation, we developed a theoretical model of movement of the polymer in the microcirculation that describes its mechanism of action during resuscitation from traumatic injury and shock. What is required for optimal water transfer out of the tissues and into the capillary space of injured tissues is the unique molecular radius that gives the polymer an intermediate osmotic reflection coefficient (σd) of 0.4-0.6, which by definition means that roughly 2/3 of the molecules stay in the capillary space and 1/3 traverse into the interstitial space. Since PEG polymers above 400 Da are cell impermeants, no molecules enter the cell. This unique intermediate σd, which is very unusual, establishes independent osmotic gradients in the microcirculation that accelerate isotonic fluid transfer. It is this unique property that causes the geometrically better performance of PEG polymers in shock, compared to either impermeant molecules only (like gluconate) or colloids only (like HES or albumin). However, since the characteristics of spinal cord tissue (and CNS in general) is much different than in the periphery, the PEG polymer sizes that have been found to be optimal in the periphery are likely too large for the CNS. We hypothesize a much smaller polymer size would be optimal and targeted polymers that display an osmotic reflection coefficient of 0.4-0.6. The work in the first year has completed those studies and analysis. We report that polymers between 1-5 kDa are theoretically optimal in spinal cord tissue. These sizes will be used in the next 2 years to affect optimal water transfer out of injured spinal cord tissue to decompress the microcirculation, enhance oxygen transfer, and ultimately improve functional cord outcomes after resuscitation and recovery from injury.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>1</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>1</td>
</tr>
<tr>
<td>4. Impact</td>
<td>10</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>10</td>
</tr>
<tr>
<td>6. Products</td>
<td>11</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>11</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>13</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>13</td>
</tr>
</tbody>
</table>
1. **INTRODUCTION:**
   In the first year we determined the optimal polymer sizes required to produce a theoretical optimized osmotic transfer of metabolic water out of the spinal cord tissues after injury and into the capillary space for rapid removal by convective transfer. In this year’s studies, we have optimized the spinal cord injury models and the polytrauma models that will be used to test the effectiveness of osmotic PEG polymer treatment.

2. **KEYWORDS:**
   Spinal cord; CNS; rodent; polyethylene glycol; osmotic reflection coefficient; pharmacokinetics; half-life of elimination; PEG-20k

3. **ACCOMPLISHMENTS:**
   
   **PEG Immunohistochemical Localization Protocols:** We identified two commercially available antibodies directed against polyethylene glycol polymers that had some validation in the literature. One antibody was directed specifically at the backbone polymer ethylene glycol repeat units in the PEG polymer. The other antibody was raised against the methoxy group of methoxy-PEG, which is a chemical derivative of PEG where a terminal methyl alcohol group is attached to the end of the polymer. To test these antibodies, rats were injected IV with either methoxy PEG with molecular weights of 550 Da or 2,000 Da. After an hour, liver and spinal cord tissues were recovered for immunohistochemical staining using the two antibodies. Liver was used as a positive control where we know these polymers will distribute based on our other work. The main objective was to see which antibody worked better so we removed the tissue of origin as a variable by starting with the staining of liver sections. The sections were prepared at 30 uM thickness on a cryostat and then permeabilized and stained with primary antibody followed by a fluorescently labeled secondary antibody directed against the rabbit IgG primary antibody. The sections were then visualized and photographed using fluorescence microscopy. In some control slices, the primary antibody was omitted to identify non-specific binding in the tissue as a background so the antibody specific staining could be determined. The results of these experiments is shown in Figure 1.
Figure 1

As can be summarized by the bar graph to the right in the figure, both backbone directed and methoxy directed primary antibodies produced significant signal above the background from the secondary antibody alone. Furthermore, the signal intensity was much greater for the smaller 550 polymer compared to the much larger 2000 Da polymer, which is consistent with the known partitioning of these sizes in tissues. The smaller 550 polymer size is a cell impermeant and distributes to the interstitial space whereas the larger 2000 Da polymer is less permeable to the sinusoids in the microcirculation. The main conclusion is that both commercially available primary antibodies to PEG work for staining PEG in tissues. Furthermore, the exact protocol steps that were used to stain the tissues also worked. We will next try this protocol and the two antibodies on spinal cord tissue in the rat.

We next repeated the previous studies but in both brain and cord tissue from uninjured rats. These results are shown in Figure 2 for cord tissue using both backbone and methoxy antibody at various titers. Again, some sections without the primary antibody were included as a control for nonspecific staining.

Figure 2.
Unlike the liver tissue, which produced strong specific binding signals for both 550 and 2000 Da PEG polymers, only the small 550 was detectable in the neuronal tissues from the CNS. However, since we know that the antibodies are capable of detecting 2000 Da PEG in tissues, these results mean that either 2000 is not getting into the spinal tissue or the antibody is not optimized to see it if it is there. From our previous work measuring osmotic reflection coefficients in the cord, we do know that PEG polymers as large as 5000 are able to escape the capillary and enter the cerebral spinal fluid. Therefore, we conclude that the most likely cause of not seeing 2000 PEG in the cord tissue is because the antibody binding is not optimized. Because we will use PEG polymers in the cord specific flush that are sized bigger than 550 (probably between 2000-5000 Da), we must increase the sensitivity of the assay to be able to detect these larger polymers in the cord tissue. We will spend some time on this as we move to the cord injury and solution-testing phase of the study. We believe that optimization of an immunohistochemical detection approach to determining PEG polymer distribution will be superior to using the fluid compartment labelling experiments that we performed in year 1 to study differential polymers distribution analysis in injured spinal cord tissues.

Spinal Cord Injury Experiments: In some of the early control spinal cord injury animals without any treatment, locomotor function was graded using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. The BBB scale uses a range from zero (no hind limb joint movements) to 21 (normal movements and coordinate gait). Before surgery, all animals are handled in the openfield maze once-daily for 7 days preceding surgery. On the first postoperative day (POD1), and at PODs 4, animals are placed in the open field and observed for 4 min. At each POD time point, all animals per group are tested. Two researchers blinded to the treatment group observed the animals in open-field testing. Hind limb movement scores were averaged to obtain a single score for each animal per time point. Mean BBB scores are tallied and plotted as a function of time after injury. The scores of a few rats are shown in Figure 3 (mean +/- SD).

Fig 3

Normal rats without surgery and surgical sham rats that underwent a laminectomy without impact injury scored normal on the BBB movement test, where a score of 21 is considered normal. A slight deficit in the laminectomized sham rats is seen in the first 2 postoperative days due to movement irregularities secondary to incisional pain and muscle detachments /scarring during the laminectomy period. Clear differences are seen in the rats with spinal cord injury (SCI) from impactor lesions created on the opened spinal
cord compared to either the normal rats or the laminectomized rats. The SCI injured rats slowly regain movement over the first 4 post-operative days. These control injury scores will be used later to assess the effects of the prototype impermeant solution given to the rats shortly after spinal cord injury.

**Metabolic Cell Swelling with Spinal Cord Injury:** In earlier studies, we determined the osmotic reflection coefficients of a variety of sizes of PEG polymers, with the goal of finding ones that have optimal water transfer properties to move water out of metabolically swollen cord tissue after injury. After identifying smaller polymers (2-5 kDa) as optimal, we began testing their water pulling power in injured spinal cord tissues by measuring total tissue water (TTW) content of the spinal cords after injury. The results of these studies is summarized in **figure 4**. Rats were given a standardized SCI injury protocol and the spinal cord tissue was removed 24 or 72 hours later for determination of TTW content. Some animals were not injured but underwent laminectomy only and some were injured and received either LR (lactated ringers solution) or PEG-20k dissolved in LR, or a cocktail of PEG-2, -5, and -20k in what we believe is an optimized LVR solution.

**Figure 4.**

We documented a 30% increase in tissue swelling or tissue water content after 24 hours after injury. The LR vehicle did not affect this degree of swelling but both LVR solutions containing PEG significantly reduced tissue swelling as indexed directly by measuring total tissue water content. Interestingly, there wasn’t a benefit with the "optimized" PEG polymer cocktail compared to PEG-20k, which is used for hemorrhagic shock.

Also of interest was the loss of effect after 72 hours from injury where the spinal cord tissue regained water weight equal to what was observed on day 1 in the untreated rats. While the treatment effect with PEG appears small, this probably reflects logarithmic decreases in intra-canal pressure and increases in spinal cord microcirculatory blood flow. This may not be identified under the experimental conditions used because a
laminectomy was performed to expose and injure the cord. We speculate that cord swelling, pressure development, and drops in capillary blood flow from microcirculatory compression would be much higher in normal field conditions where a laminectomy is not performed that allows the spinal tissue to freely swell outside of the closed case of the spinal canal. Finally, we hypothesize that combining spinal cord injury with hemorrhagic shock in our polytrauma experiments will potentiate these effects further since PEG-20k has powerful effects during shock alone to increase capillary perfusion. It is probable that hypovolemic shock and SCI potentiate injury to the cord and that the PEG treatment effect will be much greater under those real life conditions.

The return of swelling after 72 hours could be related to two events. The swelling may be metabolic in nature and reflect the still injured neurons ability to actively control their own cell volume or the later swelling may not be metabolic in nature and it may represent a secondary inflammatory response that causes capillary leak from inflammatory mediators. This extracellular swelling (edema) is different from intracellular metabolic swelling. Histological assessments are ongoing to determine the cause of this secondary swelling, which will help us understand if a second round of impermeant dosing may be effective and worth trying.

Short Term Locomotor Function in SCI:

In other studies, we began to examine motor outcomes in rats with SCI in the following groups
1.) Controls with just laminectomy surgery (no SCI)
2.) Rats with SCI
3.) Rats with SCI treated with the LR vehicle I.V.
4.) Rats with SCI treated with PEG-20k dissolved in LR, and
5.) Rats with SCI treated with a cocktail of PEG polymers, including PEG-2k, PEG-5k, and PEG-20k.

Before and at various days after spinal cord injury, rats were tested using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. The BBB scale uses a range from zero (no hind limb joint movements) to 21 (normal movements and coordinate gait). Before surgery, all animals are handled in the open-field maze once-daily for 7 days preceding surgery. On the first postoperative day (POD1), and at PODs 4, 7, 10, 14, and 21, animals are placed in the open field and observed for 4 min. At each POD time point, all animals per group are tested. Two researchers blinded to the treatment group observed the animals in open-field testing. Hind limb movement scores are averaged to obtain a single score for each animal per time point. Mean BBB scores are tallied by injured groups and plotted as a function of time after injury. Figure 5 shows some very early results in 2-3 rats per group.
secondary to the laminectomy, which is common to all groups. But, the controls rapidly regain motor control and return quickly to baseline function in 1-2 days after surgery. By contrast, the SCI alone rats show clear deficits in motor activity over 10-14 days. Generally, these animals historically will not get better after 10-14 days and their score of 8-12 on the BBB is about as good as it will get. This plateau range of scores is shown in Figure 5 by the hatched box area. The SCI rats receiving a single low volume resuscitation of 10% PEG-20k so far are doing better on this test starting at 7 days after injury, relative to the untreated groups. So far, it seems that the PEG-20k is able to provide higher levels of motor recovery of the hind limbs. More studies need to be done for longer time periods to be sure. Interestingly, the cocktail of small and large PEG polymers, which we believed may be better than PEG-20k alone, seems to show no improvement over the untreated controls.

Long term locomotor function in SCI: The results of the 8 week testing (8 weeks following initial spinal cord injury) in 5 groups of rodents is shown in Figure 6.

Our initial studies used an injury severity of a 12.5 mm drop height. This means that the open spinal cord was impacted by the SCI device impactor traveling downward onto the spinal cord from a height of 12.5 mm under the force and acceleration of gravity. This is the typical impact severity setting and the most used in the literature. In reported studies using the 12.5 mm severity level, the rats start with almost complete paralysis of the cord after one day from impact but slowly regain motor function of the cord over 4 weeks to
achieve a steady state plateau just below about 50% of normal on the BBB scale, or about a score of 10 (Fig 6). However, all of our groups showed an almost complete return of cord function after 4 weeks. Not surprising, we did not see any differences in any of the treatment groups using the 12.5 mm drop height, probably because there was no room for improvement in the model because even the vehicle treated rats fully recovered. The reason why our results are less severe compared to others reported in the literature is not clear. The technician operating the injury device does so consistently but many subjective factors can explain differences in outcomes, even when the drop height remains unchanged. Specifically, the alignment of the stopping point of the impactor on the cord and the depth it is allowed to penetrate the cord (0.5 mm) can be subjective and result in different outcomes between operators. We posit this is what happened since we use the same technical operator for all of our SCI injury studies in this project. Therefore, in an attempt to improve the resolution of the BBB assay to be able to detect changes in treatment groups, we increased the drop height to 25 mm to lower the scores that the control rats plateau at in order to provide room for improvement, should it occur. In early studies in a separate group of injured rats receiving only the LR vehicle with an injury severity of 25 mm, we observe a plateau more consistent with other studies. Five rats have been taken out to 5 weeks post-injury and show a plateau of function reflected by a BBB score of 10-11 (Figure 6, blue curve). The hatched area is where we would like to be based on historical results. As we finish taking these rats out to 8 weeks, we feel confident now that we will be able to see improvements in spinal cord function in any treatment groups using the higher severity model, should improvements actually occur.

Neurobiological evidence of SCI in 25 mm drop model: The major outcome in this model is spinal cord function as indexed by locomotor function in various tests as the rats recover after spinal cord injury. The trajectory of their recovery tells us if the new resuscitation strategies will be effective for attenuating SCI injury. These motor function curves take 2 months to perform so a lot of time and resources are invested in each rat. Therefore, we want to be certain that the exact model used is performing as it is intended to perform. Therefore, in this year, we have invested a lot of time into validating the 25 mm weight drop model, as used by our lab, will produce the anticipated outcomes in the control rats. We seem to have good motor function results on the BBB test. We also are establishing the histological and immunohistochemical outcomes that historically predict the severity of the injury. We are in the process of now analyzing all of the spinal cord tissue using these tests. Figure 7 shows the early results from a few rats. These data clearly demonstrate the anticipated anatomic changes associated with the injury site and the associated neuro-inflammation that follows, which all confirm our model severity. This gives us confidence when we move into the third year where multiple test groups will be compared to the control groups in both chronic spinal cord function testing (motor function tests) and histological outcomes.
Figure 7: Frozen sections of the rat spinal cord 72 hours after injury with immunohistochemical staining for Glial Fibrillary Acidic Protein (GFAP). GFAP is a CNS specific intermediate filament cytoskeletal protein and marker for activate astrocytes. Expression of this protein indicates the degree of inflammatory reaction to the cellular injury. Both spinal cord sections below show a large degree of GFAP expression at the site of impact injury. Panel B is from a control rat resuscitated with lactated ringers solution and panel A is from a rat resuscitated with 10% solution of PEG-20k, 5k, and 2k. The resuscitation was performed 30 minutes after spinal cord injury was induced with a 25 mm weight drop. The early analysis from a few rats in each group seems to suggest a much larger change in GFAP expression in the control compared to the PEG treated rats. Much more analysis needs to be performed on countless more sections that have been already prepared from spinal cord injured rats.

Polytrauma model development- SCI plus Hemorrhagic Shock: A major goal of this project is to determine the role of PEG polymer on spinal cord injury outcomes in both SCI alone and SCI with co-existing trauma and shock because that is likely the most common clinical manifestation of SCI in military field medicine and because the two injuries will necessarily potentiate one another. To that end, we have developed a polytrauma shock protocol using our standard rat shock protocol with modifications to accommodate an SCI insult as well. The requirements of this model is that it produce the greatest survivable traumatic and metabolic injury as possible. Survivability is key because the SCI injury component requires chronic development and monitoring. Preliminary studies show progress on a new model modified from the original lethal model by adjusting downward the amount of oxygen debt delivered during hemorrhage. We moved the lactate value that would trigger resuscitation from 10 mM to 7 mM. This requires an average blood loss of about 30% of the estimated total blood volume of the rat. The model nicely produces a severe metabolic and cardiovascular derangement that is 100% survivable on its own and after a spinal cord injury using a 25 mm weight drop model. Figure 8 below shows the arterial pressure response in the two models after the saline low volume resuscitation is administered to the rats but before a spinal cord injury is induced.
SUMMARY: In the first year we characterized the theoretical optimum polymer size for achieving the best result in moving water out of the metabolically swollen spinal cord after injury. In this current year, we have focused on documenting the model behavior in the control animals so we can be able to visualize changes in the treatment groups, should they occur. We also recalibrated the shock component of the polytrauma model that will allow us to do testing on, not only spinal cord injury per se, but also spinal cord injury in an animal with co-existing cardiovascular and metabolic trauma (shock) in a clinically relevant polytrauma model. Finally, we have shown that the rat spinal cord tissues are behaving normally following injury by their astrocyte reactivity (inflammatory injury). This establishes a firm baseline in all of our models to now test the efficacy of an optimized polymer solution on neurological injury to the cord and on cord motor function as the animals recover from spinal cord injury.

- What were the major goals of the project? There were two goals of the project;
  1. To optimize PEG polymer radius size through changes in molecular weight and arm groups until they possess favorable capillary pore permeability attributes in the spinal cord microcirculation ($\sigma_d = 0.5$) that allows for the establishment of double osmotic gradients in the microcirculation
  2. To determine the effects of optimized PEG polymer sizes on spinal cord injury as indexed by cord swelling, cord capillary blood flow, spinal cord histological markers of injury, inflammation, and post-recovery neuro-motor and functional tests.

The first goal was scheduled to be completed in the first year and the second goal in the subsequent 2 years of the project. The first goal has been completed fully at the end of the first year. We are now on to testing the newly sized polymers in the spinal cord injury models.
- **What was accomplished under these goals?** See the above section 3 (Accomplishments).

- **What opportunities for training and professional development has the project provided?** Dr. Mangino’s lab trains graduate students (MS and PhD level), undergraduate students in the Exercise Physiology program, medical student (first year), post-doctoral fellows, and general surgery residents after their second clinical year. While all of these students participated in the studies on this project to some extent, the post-docs and general surgery fellows participated the most in training from this project.

- **How were the results disseminated to communities of interest?** We have not reported any results to the community yet. The studies are still ongoing.

- **What do you plan to do during the next reporting period to accomplish the goals?** For the next year, we will thoroughly test the polymer based LVR solutions on spinal cord function in the SCI injured rats with and without polytrauma.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?** The results of the last year impact the field of resuscitation science and neuro-trauma by the discovery of optimized polymer sizes of PEG that can be tested in subsequent years to treat acute swelling after spinal cord injury in the pre-hospital setting when it does the most good. The models have been proven and established for polytrauma so we can begin testing for changes in motor function in SCI injured rats.

   - **What was the impact on other disciplines?** These works highlight how custom design of resuscitation solutions for compartment syndrome, neuro-trauma, cardiovascular shock, hemodynamic instability in the ICU, and burn resuscitation are possible by modifying and optimizing the basic platform.

   - **What was the impact on technology transfer?** The IP for this technology is currently protected by 3 patents. We are exploring whether amendments to these patents need to be filed to accommodate the new discoveries.

   - **What was the impact on society beyond science and technology?** Nothing to report

5. **CHANGES/PROBLEMS:** Nothing to report in all below sections
   - Changes in approach and reasons for change.
   - Actual or anticipated problems or delays and actions or plans to resolve them
   - Changes that had a significant impact on expenditures.
• Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
• Significant changes in use or care of human subjects
• Significant changes in use or care of vertebrate animals.
• Significant changes in use of biohazards and/or select agents

6. PRODUCTS: Nothing to report in any category below
• Journal publications: Two manuscripts are in preparation
• Books or other non-periodical, one-time publications.
• Other publications, conference papers, and presentations.
• Website(s) or other Internet site(s)
• Technologies or techniques
• Inventions, patent applications, and/or licenses.
• Other Product

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
• What individuals have worked on the project?

Name: Martin Mangino, PhD  
Project Role: PI  
Researcher Identifier:  
Nearest person month worked: 3.6  
Contribution to Project: Dr. Mangino is supervising all of the studies, analyzing data, assists with animal surgeries, and is preparing statistical analysis, reports, and manuscripts.

Name: Kirsty Dixon, PhD  
Project Role: Co-I  
Researcher Identifier:  
Nearest person month worked: 3.6  
Contribution to Project: Dr. Dixon is supervising the cord injury model studies and spinal cord histological assessments and tissue molecular work.

Name: Bruce Mathern, MD  
Project Role: Co-I  
Researcher Identifier:  
Nearest person month worked: 0.24
**Contribution to Project:** Dr. Mathern is a neurosurgeon and he attends the lab meetings to consult on clinical applications and to provide feedback on his previous studies using the spinal cord injury model in the rat.

**Name:** Nancy Lee  
**Project Role:** Tech  
**Researcher Identifier:**  
**Nearest person month worked:** 6  
**Contribution to Project:** Nancy does all of the rat spinal cord injury surgery for the project.

**Name:** Melissa Damon, BS  
**Project Role:** Tech  
**Researcher Identifier:**  
**Nearest person month worked:** 6  
**Contribution to Project:** Melissa processes all of the spinal cord tissue for analysis using immunohistochemistry, light microscopy, qPCR, and immunoblotting. Melissa left the lab in July 2019.

**Name:** Caitlin Archambault, BA, LVT  
**Project Role:** Lab Manager  
**Researcher Identifier:**  
**Nearest person month worked:** 3  
**Contribution to Project:** Caitlin manages the daily routine of the lab and orders supplies, assigns tasks, organizes data and documents, maintains the lab compliance for the project, and helps with veterinary issues for the animals.

**Name:** Anna Xu, MD  
**Project Role:** PGY2 General Surgery Resident (Research Fellow)  
**Researcher Identifier:**  
**Nearest person month worked:** 6  
**Contribution to Project:** Dr. Xu is new to the project since July 2019. She rotated into the lab from her general surgery residency where she will work on this project and others for the next 2 years. She has prior training in neurotrauma and research in this area. She will lead much of the work in the lab on this project and take direction from Dr. Mangino and Dr. Dixon.

**Name:** Jerry Maitland, BS  
**Project Role:** Lab Technician  
**Researcher Identifier:**  
**Nearest person month worked:** 6  
**Contribution to Project:** Jerry is a new research technician working on this project. He has prior experience in neurotrauma and will enter the PhD program in the fall of 2020.
● Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? None

● What other organizations were involved as partners? Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

● COLLABORATIVE AWARDS: None

● QUAD CHARTS:

Treatment of Spinal Cord Ischemia with Cell Impermeant-Based Resuscitation
SC160218
W81XWH-17-1-0602

Study/Product Aim(s)
1) To determine the optimal molecular weight PEG polymer in spinal cord microcirculation for use in spinal cord injury (SCI) models
2) To determine the effects of spinal cord-optimized PEG polymers in resuscitation solutions for treatment of SCI and complex trauma

Optimized polyethylene glycol (PEG) polymer sizes will be determined that provide an osmotic reflection coefficient in the spinal tissue of 0.5. Then, these polymers will be tested in a low volume resuscitation (LVR) crystallloid solution for the ability to prevent spinal cord injury in rat models of spinal cord injury with and without complex injury involving hemorrhagic shock. The goal is to develop a stable LVR crystallloid for pre-hospital use that both reduces spinal cord swelling injury and provides increased tolerance to the low volume state in lethal hemorrhagic shock. This increases safe transport times.

Timeline and Cost

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<td>Polymer optimization for solution</td>
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<td>Test in spinal cord injury (SCI)</td>
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<tr>
<td>Test in Hemorrhagic shock (HS)</td>
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Updated: (October 11, 2019)

Goals/Milestones
CY18 Goal – PEG Polymer size optimization
Polymer chain length (mass) and branching effects on osmotic reflection coefficient in spinal tissue to customize a CNS tissue specific LVR solution to prevent cord swelling

CY19 Goals – Test optimized polymer solutions in rat models
- Test in spinal cord injury (SCI) models
- Test in Hemorrhagic shock (HS) with PEG-20k (optimized for HS)
- Test new solution in SCI and HS models combined (polytrauma)
- Lab analytical work: Tissue processing (PCR, WB, IHC)

Comments/Challenges/Issues/Concerns: None yet noted.

Budget Expenditure to Date
Projected Expenditure: $1,069,688 (years 0-2)
Actual Expenditure: $1,066,626

9. APPENDICES: None