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Effects of Enhanced Oxygen Delivery by Perfluorocarbons in
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PRINCIPAL INVESTIGATOR:
Bruce E. Mathern, MD

CONTRACTING ORGANIZATION:
Virginia Commonwealth University Health System

Richmond, VA 23298-0631

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14. ABSTRACT As the incidence of spinal cord injury has increased in the combatant military population, improved methods of treating this clinical condition are necessary. Given our previous work with measuring the pO2 in CSF and the parenchyma, following the administration of perfluorocarbons in an acute spinal cord injury model, we elected to further the study by performing behavioral studies, and histopathology at multiple time points post injury. Our aim is to determine if enhanced oxygen delivery in spinal cord injury will improve functional recovery in a rodent spinal cord injury model. We hypothesize that the delivery of perfluorocarbons will diminish the loss of spinal cord parenchymal tissue, and improve ambulation and sensory function. We hypothesize that markers of cell degeneration and apoptotitis will be decreased as well.					
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Introduction

Spinal cord injury (SCI) is a biphasic process, consisting of a primary injury at the time of trauma and a secondary injury that leads to loss of tissue and motor functions.^{1,2} Following spinal cord injury damage to the regional network of blood vessels play significant factor in post-impact ischemia.^{3,4} The primary impact of SCI has been shown to cause significant mechanical damage to the parenchyma, in particular the microvasculature leading to plasma fluid leaks, reduction of cord blood flow (SCBF) and decreased oxygen and ischemia-hypoxia.¹⁻⁴ The ischemic milieu surrounding the injured cord elicits a secondary injury complex cascade and increases the expression of cell death markers as well as the activation of caspases which translate into cellular and neuronal death and worse neurological outcome.⁵⁻⁷ A linear relationship between severity of SCI and reduction in SCBF has been established, linking post-traumatic ischemia to axonal dysfunction.⁸ Decreased oxygen level in severe traumatic injuries appears to be implicated in poor functional outcome and death, a number of therapies have been targeted at enhancing oxygen delivery to the injured spinal cord in hopes of limiting the cascade of ongoing damage which is at least in part mediated by anaerobic metabolism and lipid peroxidation. Historically, ventilation with 100% O₂ has been shown to raise O₂ levels in CSF, but parenchymal levels respond minimally, in addition a significant delay in therapy is associated with increased residual deficits.⁹ To improve overall cell survival and functional outcome and provide new therapies for spinal cord injury it is essential to rapidly and efficiently enhance oxygen delivery to the injured cord. One promising candidate for SCI treatment is intravenous Perfluorocarbon (PFC). PFC's are inert small particles with highly efficient O₂ solubility and able to deliver oxygen to compromise ischemic neural tissues and they have been shown to reach areas of poor perfusion, making these molecule suitable for efficiently delivering oxygen and hold the promise of delivering therapy at the time of injury without the need burdening logistical. In animal traumatic injury studies and clinical trials it was shown that PFC's treatment resulted in better tissue oxygen consumption, less cell death, and better functional outcome.^{10,11} In recent studies we tested the efficacy of Oxycyte a new generation of PFC's in rodent spinal injury model and we have shown that Oxycyte enhances oxygen delivery to injured tissues following SCI.¹² PFC's was also shown to preserve tissue and enhance motor function in traumatic brain injury models.¹³ Therefore, we hypothesize that a dose of perfluorocarbon given intravenously after injury will significantly improve parenchyma O₂ level and will significantly decrease neuronal and perineuronal cell death and apoptosis, and promote improved functional outcomes. To test this hypothesis, we proposed to: (1) determine the effect of enhanced oxygen delivery via oxycyte emulsion on lesion size and cellular death markers in a rodent weight drop traumatic spinal cord injury model; (2) determine if enhanced oxygen delivery in spinal cord injury spares cellular elements, white matter tracts or a combination as a mechanism of action; and (3) determine if enhanced oxygen delivery in spinal cord injury improves functional recovery in a rodent injury model. The results from this research studies will elucidate the role of Oxycyte in enhanced oxygen delivery, and neuroprotection in acute spinal cord injury. These studies could present a novel new therapeutic approach to prevent tissue and neurological damage in humans post traumatic injury.

Body

These studies have the following aims: (1) determine the effect of enhanced oxygen delivery on lesion size in a rodent weight drop traumatic spinal cord injury model; (2) determine if enhanced oxygen delivery in spinal cord injury spares cellular elements and white matter tracts and the mechanisms of protection; and (3) determine if enhanced oxygen delivery could prevent neuronal loss and protects locomotor patterns and improves functional recovery in a rodent impact spinal cord injury model. Over the past 12 months, we have made significant progress on the completion of the specific aims in the scope of work. Currently all of the animal surgeries

have been completed and the cords harvested and preserved. The functional outcomes of BBB and incline table score have been completed as well and the results are currently with biostatistics to look at statistical significance. Selected spinal cords in all time points have been sectioned and have undergone immunohistochemical analysis, lesion volume measurement as well as evaluation and quantification of cell death which has allowed us to have some preliminary data. Cord sectioning and staining continues on the remaining cords. We have obtained some interesting preliminary data as outlined below.

Aim 1: Determine the effect of enhanced oxygen delivery on white matter and myelin preservation following spinal cord injury.

This study will determine how enhanced oxygen delivery affects tissues, white matter and neuron after SCI. Specifically if enhanced oxygen delivery spares cellular elements, white matter tracks, and reduces lesion size in rodents subjected to spinal cord injury. These experiments will demonstrate the effectiveness of perfluorocarbon (Oxycyte) in preserving neurons and tissues. Given the data published by us and others demonstrating that Oxycyte enhances oxygen level in hypoxic tissue after SCI and the published data showing perfluorocarbon can protect diverse central nervous system after traumatic injury, we anticipated these experiments would reveal Oxycyte role in tissue and neuronal protection after injury to the spinal cord.

1a. Research approach: Within our work proposal we performed a mid-thoracic spinal cord contusion injury using aseptic techniques. NYU (MASCIS) Impactor (Basso DM et al., 1996; Schroeder et al., 2008)^{10,11} was used to perform a T9-10 mid-thoracic spinal cord contusion injury. All studies were performed with Long-Evans Hooded Rats. We randomly divided 120 animals into four groups for survival time of 1 to 42 postoperative days: Group 1, control, unlesioned animals (n=5 per group) Group 2, contusion injury followed by intraperitoneal vehicle injection (sterile saline, n 5); Group 3, contusion injury followed by intravenous I.V. injection (2ml/kg oxycyte +O₂, n 5), Group 4, contusion injury followed by intravenous I.V. injection of sterile saline+O₂, n 5). Animals in each group were sacrificed by deep anesthesia at 1, 4, 7, 14, 21, and 42 days postoperative. Spinal cords were harvest from groups trans-cardiac perfused with 4% paraformaldehyde. Spinal cord collected and cryosectioned for further hisochemical analysis to determine white-matter and neurons sparing. These histochemical techniques allow thorough analysis of lesion severity independent of animal behavior. Sectioned cords were stained with Luxol Fast Blue to determine myelin and white matter preservation and lesion size in the 4 groups. We predicted that enhanced oxygen delivery after SCI would reduce lesion size and preserve white matter and myelin.

1b. Progress: We have made significant progress on this aim. Two week before experiment, animals were allowed to freely explore the open field. Animals were sacrificed and spinal cord isolated and sectioned. Luxol blue

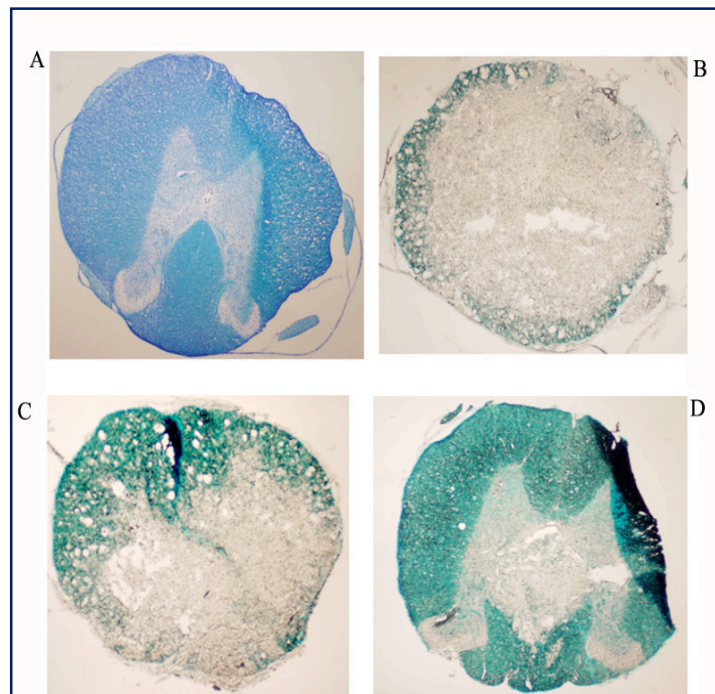


Figure 1. Oxycyte preserves myelin and white matter after SCI. Luxol Blue staining of spinal sections near the lesion epicenter A. animals sustained laminectomy; B. animals sustained SCI; C. SCI animals treated with saline control; D. SCI animals treated with 2ml/kg oxycyte emulsion

staining was performed to determine white matter preservation and demyelination in the Oxycyte groups compared to control groups.

1c. Preliminary Data: Spinal cord histochemical analysis sections were imaged and photographed. Spinal cord injured animals treated with Oxycyte emulsion combined with oxygen breathing showed better myelin and white matter preservation compared to injured control groups. As shown in (figure 1 and 2). As hypothesized spinal cord sections from injured animals treated with oxycyte showed less

myelomalacia cavities and preserved myelin. Spinal cord lesion area was significantly reduced by Oxycyte treatment. Spinal cords from rats 7 days postoperative were sectioned and stained as described in aim 1a. Animals group treated with Oxycyte after SCI had significantly better spared white matter ($p=0.0015$) compared to the SCI injury control and saline vehicle group. White matter in caudal sections from animals received Oxycyte was spared better than saline treated animals group. In the lesion epicenter there was no significant difference of white matter preservation between saline and Oxycyte group.

1d. Interpretation: Ischemia/hypoxia plays an essential role in the pathogenesis of spinal cord injury. Therefore, efficient oxygenation delivered to hypoxic tissues would result in less injury and better tissue preservation. Thus these results are in agreement with our previous publications (Schroeder et al., 2008) demonstrating that Oxycyte efficiently enhanced oxygen level in the ischemic tissues after SCI and this correlates with better white and grey matter sparing.

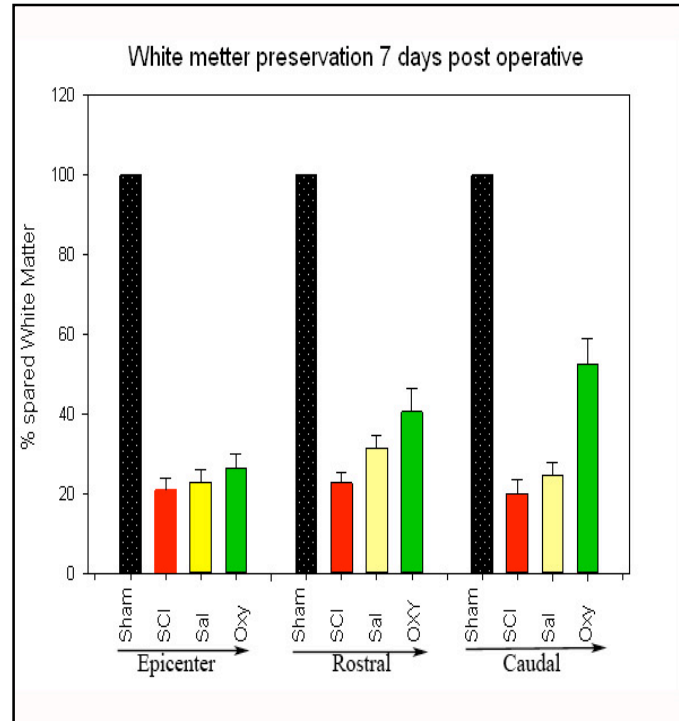


Figure 2. Levels of spared white matter in oxycyte-treated animals significantly increased after injury ($p=0.0015$) compared with control SCI animals. White matter of spinal cord lesion caudal to the epicenter area was significantly preserved by oxycyte treatment.

Aim 2: Effects of enhanced oxygen delivery on cellular and neuronal survival after spinal cord injury.

The experiments for this specific aim were designed to determine the effect of Oxycyte on spinal cord cell survival and tissue protection following SCI. The hypothesis is improving oxygen level to the injured tissue would prevent neuronal and oligodendrocytes cell death caused by hypoxia. Given that Oxycyte treatment has demonstrated better white and grey matter protection after SCI, we anticipated that Oxycyte protection correlates with less cell death.

2a. Research approach: Apoptosis-induced neuronal and glial cell death has been reported to occur in the spinal cord following traumatic injury.¹⁵ In the present study, we examined the effect of Oxycyte administration on the number of apoptotic cell death in neurons and oligodendrocytes determined by immunohistochemical analysis using Terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick-end labeling (TUNEL) staining. This staining was used as a marker of apoptosis or cell damage. Oxycyte or saline (2ml/kg) were administered immediately after contusion. Animals (n 5 per

group) were euthanized at 4, 7, 14, 21 and 42 days postoperative, followed by cords cryoprotection and 20 μ m sections, and subsequently sections were processed through graded alcohols, stained for TUNEL positive cells. Dead TUNEL positive cells were counted from section rostral and caudal to the epicenter of the contusion.

2b. Progress: We have made significant progress on this aim. Selected spinal cords in all postoperative time points have been sectioned, cryosectioned and have undergone immunohistochemical analysis. TUNEL staining was performed to calculate the average number of apoptotic cell death after SCI. Oxycyte group data were compared to the control SCI groups.

2c. Preliminary data: To determine the mechanisms by which Oxycyte preserves tissue and white matter after SCI, spinal cord sections from animals group were TUNEL immunohistochemical stained and sections were imaged and photographed. The number of TUNEL positive cells was counted in five randomly chosen fields. The immunohistochemistry analysis of sections caudal and rostral to the lesion center showed that animals given intravenous injection of Oxycyte after SCI have significantly less apoptotic cell death compared to saline alone or injured animals group (figure 3). The maximum TUNEL positive cells were observed at 7 days postinjury. TUNEL positive cells decreased significantly in lesions of Oxycyte-treated animals compared to SCI injured-untreated animals. At 14 and 21 days post-SCI, apoptotic cell also decreased significantly in lesions of Oxycyte-treated animals compared to untreated-SCI animals. There was no major significant difference of apoptotic cells in the epicenter lesions of injured animals treated with saline or Oxycyte (figure 4 and 5). These results may be due to the severe damage caused to tissue in the epicenter of injury. In the other hand oxycyte-treated group had

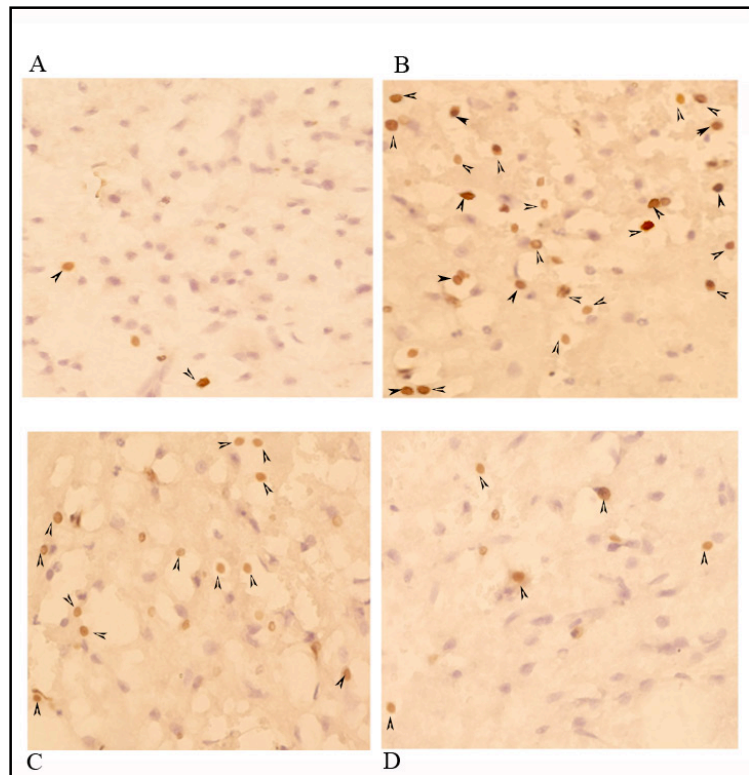


Figure 3. Oxycyte improves cell survival. A to D a representative TUNEL staining photomicrographs showing cross-sections 2 mm caudal side of epicenter lesion. All micrographs are $\times 400$ magnifications. Panel **A**. sham section, **B**. SCI control. **C**. saline treated animals. **D**. Oxycyte treated animals. Arrows indicate apoptotic cells.

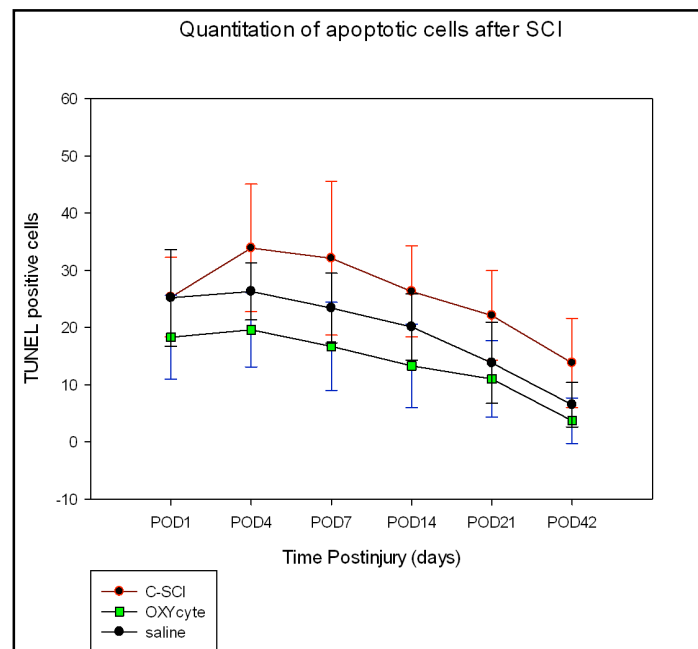


Figure 4. TUNEL positive cells decreased significantly in lesions of oxycyte-treated animals compared to SCI-untreated animals. The number of apoptotic cell death at 6 times point postinjury of spinal cord. Data are mean $\pm$ SD, $P < 0.05$

significantly less apoptotic cells death in sections 2mm rostral ($p=0.0033$) and caudal ($p=0.0001$) to center of injury compared saline group. Animals treated with Oxycyte showed reduced cell death in tissue rostral and caudal to the lesion epicenter shown in the graph (figure 5), but in the epicenter of the lesion there was no statistical difference in the number of apoptotic cells counted in the Oxycyte and saline group.

2d. Interpretation: These preliminary data showed at 7 days after injury, for both Oxycyte and saline groups, the number of apoptotic cell did not change much at the center of injury, but a tendency of increase was observed in sections of rostral and caudal to the center of injury. The greater decreases of apoptotic cell

death by Oxycyte compared to saline group were observed 2 mm caudal to the center of the lesion epicenter which suggest that Oxycyte may have ability to deliver oxygen beyond damaged tissue, where saline and O₂ probably couldn't reach further caudal tissues to the center injury. These data agree with the white matter and myelin preservation in oxycyte-injured animals group as shown in figure 1 and 2. These results indicate that Oxycyte has a neuroprotective effect following SCI. Oxycyte protection following SCI maybe due in part to attenuating apoptotic cell death and/or suppressing the onset of delayed cell death. These observations of time dependent cell death and the high number of apoptotic cells at the center of injury, but lower in regions rostral and caudal to the center may reflect the biphasic nature of the pathophysiology of the secondary injury cascade and Oxycyte treatment could be involved in delayed or attenuated secondary injury onset response. The decrease of apoptotic cell death by Oxycyte may reduce myelin damage in SCI, and might be an indication of better recovery of neurologic function.

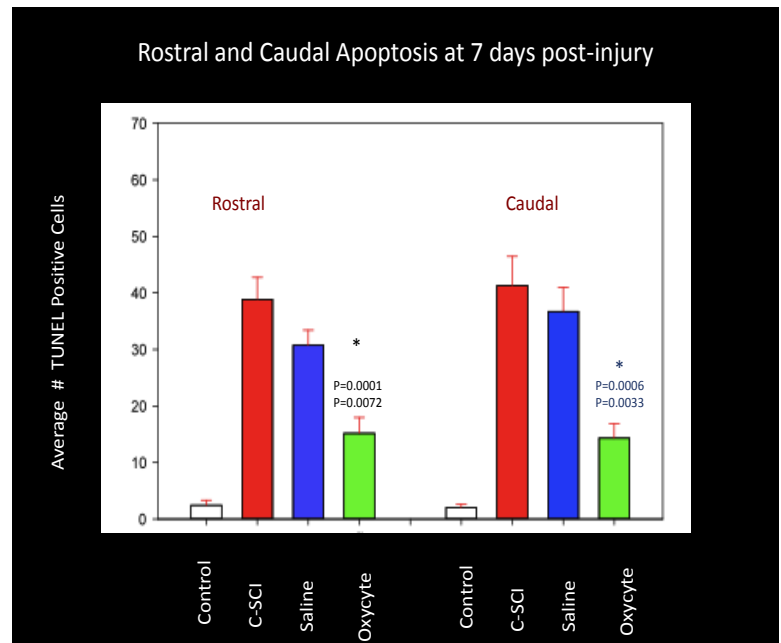


Figure 5. Oxycyte effects on cell death after SCI. The apoptotic cell deaths were significantly decreased in spinal cord sections of animals treated with oxycyte following SCI. Apoptotic cell death were analyzed by TUNEL staining of spinal cord sections from animals received oxycyte+O₂, saline+O₂, sham and SCI controls. Graph presenting mean of apoptotic cell death in lesions Rostral and Caudal to the epicenter injury. Bars represent mean values (± S.E.M).

Aim 3: Determine if enhanced oxygen delivery could prevents neuronal loss and protects locomotor patterns and improves functional recovery in a rodent impact spinal cord injury model.

The experiments of this study was to evaluate the effects of Oxycyte at 2ml/kg on motor functional recovery following SCI. We recently demonstrated in rodent spinal cord injury model that administration of PFCs combined with 100% O₂ can reverse tissue hypoxia and could be a promising therapy for reducing ischemic injury and improving functional recovery after SCI. Given that Oxycyte treatment resulted in less cell death and better tissue preservation following SCI. We anticipated that injured animal treated with Oxycyte will have improved locomotor function.

3 a. Research approach: According to the research proposed we performed a mid-thoracic spinal cord contusion injury using aseptic techniques. Contusions were performed on T9-10. All

studies were performed on randomly divided 120 animals into four groups for behavioral studies: Group 1, sham, animals (n=5 per group) Group 2, contusion injury followed by intraperitoneal vehicle injection (sterile saline, n 5); Group 3, contusion injury followed by intravenous I.V. injection (2ml/kg oxycyte +O₂, n 5), Group 4, contusion injury followed by intravenous I.V. injection of sterile saline+O₂, n 5). Locomotor function was evaluated using the Basso–Beattie–Bresnahan¹⁶ (BBB) locomotor rating scale in an open field for 4 min (Basso et al., 1995). The sham operation consisted of laminectomy without spinal cord injury. Functional tests were performed before the injury, and weekly during the study period of 1, 4, 7 14, 21 and 42 days 6 weeks.

3 b. Progress: We made significant progress on this aim. All animal surgeries and all functional measurements were completed: Behavioral and locomotor tests (BBB) and incline plane testing by two blinded evaluators. All the studies were done as outlined in the research proposal.

3 c. Preliminary data: Locomotor activity following mid-thoracic spinal cord contusion injury was assessed by Basso scale as described above (Figure 6). All animals groups showed the same locomotor dysfunction 1 day postoperative and improvements were observed thereafter. There was locomotor improvement for the Oxycyte and saline + O₂ groups compared to untreated SCI animals groups. There was no significant difference in locomotor function between the Oxycyte and saline groups.

3 d. Results interpretation: We have shown that administration of PFCs combined with 100% O₂ can reverse tissue hypoxia and we anticipated this treatment would improve locomotor function following SCI. Oxycyte administration of (2 ml/kg) following SCI was beneficial and produced significant locomotor improvements compared to the contused animals that have not received any treatment but no major difference was observed when compared to the saline+O₂ group. The statistical work is not fully investigated. Therefore, this effect on motor functional recovery requires further statistical analysis. The results of the BBB scoring and incline table performance are currently being reviewed for statistical significance. One issue identified is that several animals did not have post injury day 1 BBB scores which would be consistent with a severe injury and we are trying to determine if exclusion criteria should be applied. Dr. Wen from biostatistics is working with us to review the functional data and statistical conclusions should be available for the next quarterly technical report.

Recovery of Locomotor Functions days post injury

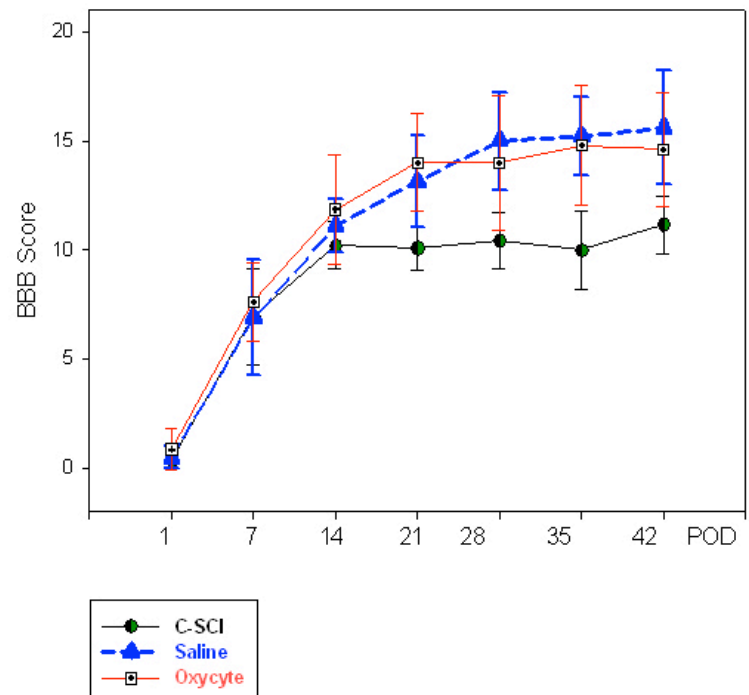


Figure 6. BBB locomotor activity for the different groups following SCI, at 1 to 42 postoperative days in. Scores reveal significant hindlimb dysfunction in SCI untreated group. From day 21 both saline and Oxycyte treated groups showed increase in movement of their hindlimbs compared SCI. A higher score number represents greater function recovery. Error bars represent the mean of standard error ($\pm$ S.E.M).

Ongoing experiments: We are continuing to perform the immunohistochemistry experiments for cell and neuronal death markers (FAS, TNF immunohistochemical staining), and the detailed statistical analysis for all animals groups to enhance the significance of the preliminary data.

Comparison and evaluation of immunohistochemical results with appropriate correlation to the locomotor function analysis studies.

Key Research Accomplishments:

- New Personnel Hired: Dr. Yacoub
- All animal surgeries complete and all cords harvested and available for sectioning and staining.
- All Functional measurements completed: BBB and incline plane testing by two blinded evaluators
- Preliminary data on white matter staining and apoptosis/tunel staining available
- Dr. Hajec reported preliminary results to the Neurosurgical Society of the Virginias meeting Jan 2012.

Reportable outcomes:

This research proposal has resulted in Dr. Hajec preliminary results presentation to the Neurosurgical Society of the Virginias meeting Jan 2012.

At this point, we have included the preliminary results. However, based on our recent research data and the ongoing work, it is expected that this work will produce at least one publication, will be presented at a national meeting.

Conclusion

This research strategy focused on determining the potential therapeutic benefits of Oxycyte emulsion in a clinically relevant model of rat spinal cord contusion injury. Oxycyte treatment was obviously able to enhance oxygen delivery to the hypoxic tissues and suppress lesions size and apoptotic cell death predicting locomotor function improvement. Though statistical analysis continues and full data on all spinal cords is not available, preliminary data show that perfluorocarbon treated animals had statistically significant reductions of apoptosis rostral and caudal to the injury site, preservation of white matter tracts caudal to the injury lesion. Functional measures of BBB and incline tilt are trending towards improved outcome with perfluorocarbon treated animals but exclusion analysis and statistical analysis is ongoing. We are on schedule with the revised scope of work submitted with the no cost extension and look forward to concluding the work on this project. These research studies will lead to submission of manuscripts and additional grant proposals.

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