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Effects of Enhanced Oxygen Delivery by Perfluorocarbons in  
Spinal Cord Injury

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<b>14. ABSTRACT</b> 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 apoptosis will be decreased as well.					
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## Introduction

A goal of the studies is to develop a novel therapy for spinal cord injury (SCI). In our current research initiative we evaluated the potential of Perfluorocarbons as an agent that is easily deployed and administered even in the most remote setting. Our previous studies indicate that early administration of Perfluorocarbons decrease hypoxia in the tissue surrounding the spinal cord injury. Spinal cord injury 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.<sup>1,2</sup> Following spinal cord injury damage to the regional network of blood vessels plays a significant factor in post-impact ischemia.<sup>3,4</sup> 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.<sup>1-4</sup> 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.<sup>5-7</sup> A linear relationship between severity of SCI and reduction in SCBF has been established, linking post-traumatic ischemia to axonal dysfunction.<sup>8</sup> 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% O<sub>2</sub> has been shown to raise O<sub>2</sub> levels in CSF, but parenchymal levels respond minimally, in addition a significant delay in therapy is associated with increased residual deficits.<sup>9</sup> 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 O<sub>2</sub> solubility and able to deliver oxygen to compromise ischemic neural tissues and they have been shown to reach areas of poor perfusion, making these molecules 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.<sup>10,11</sup> 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.<sup>12</sup> PFC's were also shown to preserve tissue and enhance motor function in traumatic brain injury models.<sup>13</sup> PFC's have been studied extensively for safety in normal humans and have recently been used at our institution in a pilot trial of 9 severely head injured patients with encouraging results. Therefore, we hypothesize that a dose of perfluorocarbon given intravenously after injury will significantly improve parenchyma O<sub>2</sub> 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 these 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 with the long-term goal of using PFC in a spinal cord

population to determine efficacy and safety trial followed by a phase III clinical trial to evaluate proposed functional improvement following PFC administration.

## **Body**

As described above, several studies have shown that ischemia/hypoxia play crucial role in the devastating effects of the secondary injury following SCI which translates into worse neurological outcome and larger lesion size. The principal goal of the project is to protect spinal cord tissue and preserve neuronal function after SCI. The development of a rapid and efficient oxygen delivery may enable to halt the hypoxic milieu initiated after the primary mechanical injury, with the long-term translational goal of reducing the secondary injury effects and improving neuronal and motor function. As a first step in this developmental project, we proposed the following exploratory aims.

**Aim 1:** Determine the effect of enhanced oxygen delivery on lesion size in a rodent weight drop traumatic spinal cord injury model

**Aim 2:** Determine if enhanced oxygen delivery in spinal cord injury spares cellular elements and white matter tracks and the mechanisms of protection

**Aim 3:** Determine if enhanced oxygen delivery could prevent neuronal loss and protect locomotor patterns and improve functional recovery in a rodent impact spinal cord injury model.

### **Tasks Involved in Accomplishing Aim 1:**

- 1) Animal surgeries have been completed and the cords harvested and preserved.
- 2) Spinal cords in all time points have been sectioned and have undergone immunohistochemical analysis, lesion volume and white matter sparing.
- 3) Statistical analysis of lesion volume and white matter sparing were done.

**Materials/Methods/Outcomes (Aim 1, 2 and 3): (Please see Appendix for our manuscript with figures, in press in; J Neurotrauma. 2013 Sep 12. [Epub ahead of print] PMID: 24025081; PubMed - as supplied by publisher)**

- 1. Determine the effect of enhanced oxygen delivery on white matter and myelin preservation following spinal cord injury:** Ischemia/hypoxia plays an essential role in the pathogenesis of spinal cord injury and we have shown that Oxycyte efficiently enhanced oxygen level in the ischemic tissues after SCI (Schroeder et al., 2008). Therefore, efficient oxygenation delivered to hypoxic tissues would result in less injury and better tissue preservation.

**1 a. Research approach:** This study was designed to 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. 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)<sup>10, 11</sup> was used to perform a T9-10 mid-thoracic spinal cord contusion injury. 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 (5ml/kg oxycyte +O<sub>2</sub>, n 5), Group 4, contusion injury followed by intravenous I.V.

injection of sterile saline+O<sub>2</sub>, 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.

**1 b. Research results:** Using Oxycyte to enhance oxygen delivery after SCI significantly reduced myelomalacia cavities, reduced lesion size and preserved myelin (**Data and statistical analysis are described in appendix**).

### **Tasks Involved in Accomplishing Aim 2:**

- 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. Our hypothesis is improving oxygen level to the injured tissue could prevents neuronal and oligodendrocytes cell death caused by ischemia/hypoxia. Given that Oxycyte treatment have demonstrated significant better white and grey matter protection after SCI, we anticipated that Oxycyte tissue protection will correlates with less cell and neuronal death.

**2a. Research approach:** Apoptosis-induced neuronal and glial cell death has been reported to occur in the spinal cord following traumatic injury.<sup>15</sup> In the present study, we examined the effect of Oxycyte administration on the number of apoptotic cell death in neurons and oligodendrocytes were 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 µm 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. Research results:** The data of TUNEL positive cells 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. 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. (**Data and statistical analysis are described in appendix**).

### **Tasks Involved in Accomplishing Aim 3:**

- 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:** The experiment of this study was to evaluate the effects of Oxycyte at

5ml/kg on motor functional recovery following SCI. We have demonstrated in rodent spinal cord injury model that administration of PFCs combined with 100% O<sub>2</sub> 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:** 120 animals were randomly divided 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 +O<sub>2</sub>, n 5), Group 4, contusion injury followed by intravenous I.V. injection of sterile saline+O<sub>2</sub>, n 5). Locomotor function was evaluated using the Basso–Beattie–Bresnahan<sup>16</sup> (BBB) locomotor rating scale in an open field for 4 min (Basso et al., 1995) and inclined table performance. 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. Research results:** PFCs combined with 100% O<sub>2</sub> improved locomotor function following SCI in Oxycyte and saline groups. **(Data and statistical analysis are described in appendix).**

#### **KEY RESEARCH ACCOMPLISHMENTS:**

1. All animal surgeries complete and all cords harvested, sectioned and stained.
2. All Functional measurements completed: BBB and incline plane testing by two blinded evaluators
3. White matter staining and apoptosis/TUNEL staining done and analyzed
4. Dr. Hajec presented the PFCs data to the Neurosurgical Society of the Virginias meeting Jan 2012.
5. Medical student trained in spinal cord injury during the summer of 2013.
6. The manuscript with figures and conclusions were prepared, submitted, revised and actually in press in **(Journal of Neurotrauma; 2013 Sep 12. [Epub ahead of print] PMID: 24025081; PubMed - as supplied by publisher).**

#### **Reportable outcomes:**

This research proposal has resulted in Dr. Hajec presentation to the Neurosurgical Society of the Virginias meeting Jan 2012. We have generated a manuscript of our findings and it is in press (Journal of Neurotrauma. 2013 Sep 12. [Epub ahead of print] PMID: 24025081; PubMed - as supplied by publisher). This published manuscript is included in the appendix for a detailed description of all materials, methods, and findings related to the use of PFCs and its neuro-protective property after spinal cord injury.

## Conclusion

Since initiation of funding, we have accomplished all of the goals outlined in the original statement of work. Additionally, we have initiated a research study to determine the efficacious neuroprotective dose of PFCs following SCI and we generated a manuscript published in the *Journal of Neurotrauma*. The foundation of work enabled by this grant will enable future funding to continue investigating Perfluorocarbon dose efficacy and to initiate clinical trials for human with spinal cord injury.

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**APPENDICES: Manuscript entitled “Neuroprotective Effects of Perfluorocarbon (Oxycyte) after Contusive Spinal Cord Injury”** (doi: 10.1089/neu.2013.3037). *Journal of Neurotrauma*

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**<http://www.ncbi.nlm.nih.gov/pubmed/24025081>**

## Neuroprotective Effects of Perfluorocarbon (Oxycyte) after Contusive Spinal Cord Injury

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**Key words:** SCI; ischemia/hypoxia; perfluorocarbon (Oxycyte); oxygen therapy; neuroprotection

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## **Abstract**

Spinal cord injury (SCI) often results in irreversible and permanent neurological deficits and long-term disability. Vasospasm, hemorrhage and loss of microvessels create ischemic environment at the site of contusive or compressive spinal cord injury and initiate the secondary injury cascades leading to progressive tissue damage and severely decreased functional outcome. Although the initial mechanical destructive events cannot be reversed, secondary injury damage occurs over several hours to weeks, a time frame during which therapeutic intervention could be achieved. One essential component of secondary injury cascade is the reduction in spinal cord blood flow with resultant decrease in oxygen delivery. Our group has recently shown that administration of fluorocarbon (Oxycyte) significantly increased parenchymal tissue oxygen levels during the usual post injury hypoxic phase and fluorocarbon has been shown to be effective in stroke and head injury. In the current study, we assessed the beneficial effects of Oxycyte after a moderate to severe contusion SCI was simulated in adult Long-Evans hooded rats. Histopathology and immunohistochemical analysis showed that the administration of 5ml/kg Oxycyte PFC (60% emulsion) following SCI dramatically reduced destruction of spinal cord anatomy and resulted in a marked decrease of lesion area, less cells death and greater white matter sparing at 7 and 42 days post-injury. TUNEL staining showed significant reduced number of apoptotic cells in Oxycyte treated animals compared to saline group. Collectively these results demonstrate the potential neuroprotective of Oxycyte treatment following SCI and its beneficial effects maybe in part due to reducing apoptotic cell death and tissue sparing. Further studies to determine Oxycyte efficacious dose and its mechanisms of protections are warranted.

## Introduction

Spinal cord injury (SCI) causes life-long disability and often permanent neurological deficits.<sup>1</sup> Despite advances in clinical research and rehabilitation strategies, there are still no effective treatment options to significantly improve functional recovery following SCI.<sup>2-4</sup> Spinal cord injury consist of a primary injury at the time of trauma followed by a series of destructive cellular processes, known as secondary injury cascade leading to loss of tissue and motor functions.<sup>5-10</sup> Though the pathophysiology of SCI is not fully understood there is general consensus that the primary impact causes significant mechanical damage to the parenchymal microvasculature leading to disturbances of spinal cord blood flow (SCBF), severely reduced oxygen levels, perfusion defects, hemorrhage, ischemia, and hypoxia.<sup>9,11-18</sup> Among all of secondary injuries, ischemia/hypoxia has been demonstrated as a focus of postinjury pathophysiological changes of acute SCI since it elicits the 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.<sup>8, 9,19-21</sup> Therefore, improving spinal cord tissue oxygenation is a logical and important strategy in the treatment of SCI. A linear relationship between severity of SCI and reduction in SCBF has been established, linking post-traumatic ischemia to axonal dysfunction.<sup>8, 22</sup> 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% O<sub>2</sub> has been shown to raise O<sub>2</sub> levels in CSF, but parenchymal levels respond minimally,<sup>23, 24</sup> in addition a significant

delay in therapy is associated with increased residual deficits. One way to overcome such obstacles would be the use of agents or strategies that could rapidly and efficiently enhance oxygen delivery at the time of injury, or shortly thereafter to minimize the ischemic injury. One promising candidate with good safety and tolerability is intravenous Perfluorocarbon (PFC).<sup>25,26</sup> PFCs are non-toxic oxygen carriers with an unrivalled capacity to dissolve gases including oxygen. They are inert fluorinated hydrocarbons with highly efficient O<sub>2</sub> solubility and small particle size <0.2 μm. PFC's ability to increase O<sub>2</sub> delivery is based on its O<sub>2</sub> linear dissociation curve making these molecule suitable for efficiently delivering oxygen to compromised ischemic neural tissues and can penetrate microcirculation of poor perfusion, and hold the promise of delivering therapy at the time of injury without the need of burdening logistics.<sup>27</sup> A number of animal traumatic injury studies and Phase III clinical trials conducted on such emulsions have shown that they can help in preventing the risk of oxygen deficiency in tissues, and have shown beneficial effect in both stroke and head injury.<sup>28-32</sup> Oxycyte an injectable oxygen carrier is a new generation of PFC's which have better property and fewer side effects was developed by (Oxygen Biotherapeutics Inc., Durham, NC). Oxycyte is a 60% emulsion of perfluoro-tert-butylcyclohexane (FtBu) with phospholipid emulsifiers. In several large and small animal models PFC's was shown to preserve tissue and enhance motor function after traumatic brain injury,<sup>29,33</sup> but its potential therapeutic in traumatic spinal cord injury has not been explored. On the basis of these considerations we tested the hypothesis that the enhanced oxygen delivery could minimize the secondary damaging processes and loss of function following SCI. To test this idea we have previously shown that the pressure of oxygen levels dissolved in the blood in spinal cord injury dropped profoundly from 21.4 to 10.4 mm Hg almost immediately post injury and all animals that received Oxycyte combined with 100% oxygen showed significant improvement, with a mean increase in oxygen levels of 23.3 mm Hg.<sup>34</sup> This data confirmed that Oxycyte is effective vehicles for oxygen delivery after SCI, and can significantly

increases tissue oxygen levels during the post injury hypoxic phase.<sup>34</sup> Although our previous data established that Oxycyte improves tissue oxygen level in hypoxic parenchyma after SCI, Oxycyte's effects on tissue and neuronal preservation in experimental models of SCI have been not reported. In the present study, we explore the role of Oxycyte in a rat model of SCI and we hypothesized that a dose of perfluorocarbon given intravenously after injury will significantly decrease neuronal and peri-neuronal cell death and apoptosis, and promote improved functional outcomes. Here we report that Oxycyte reduces spinal cord damage and improves tissue sparing by directly reducing the lesion size and neuronal cell death. Our findings support the growing body of evidence that PFCs such as Oxycyte may be neuroprotective and further substantiate the use of PFC as a potential therapeutic strategy for acute SCI.

## **Materials and Methods:**

### **Animal model and surgical procedures**

Long-Evans rats (Harlan, Indianapolis, IL) 11-13 weeks old weighing between 220 to 250g, were used for spinal cord injury protocols. All housing, surgical and postoperative care procedures were performed in accordance with the Department of Defense, and Virginia Commonwealth University Institutional Animal Care and Use Committee. Animals were kept on a 12 hour light/dark cycle, and acclimatized for one week prior to surgery with feed, water, handling and the behavioral methods. The animals underwent anesthesia induction with 5% isoflurane and were maintained with 2.5% isoflurane during all surgical procedures. Depth of anesthesia was tested priorly and throughout the surgical procedures by heart and respiration rate, and toe pinch. A rectal temperature probe was placed and the animal laid on a homeothermic blanket to maintain normothermia (37.0C +/-1). Animals received pre-incision analgesia with buprenorphine. The anterior left neck, rostral chest, and dorsal thoracic region were shaved, prepped and draped for surgery. Animals were initially laid supine. Using aseptic technique, a

left jugular vein exposure was completed, and then the animal was placed in a stereotactic frame. A dorsal midline incision was made over the mid-thoracic spine, using the T5 prominence as a guide. Paraspinal muscles were carefully dissected off the spinous process, and lamina. A standard T 9/10 laminectomy was performed using micro-rongeurs to make the spinal cord and exposed dura ready for cord contusion. The animal was then placed on the MASCIS II-NYU Impactorplatform<sup>35,36</sup> and secured using clamps attached to the spinous processes of T8 and T11, above and below the laminectomy to produce a standardized graded weight drop contusion. The impactor weight device (10grams) was dropped from a height of 25mm (Range 6.25-50mm), producing a moderate-severe cord contusion-spinal cord injury, resultant in hind limb paralysis and neurogenic bladder. The impactor rod was removed immediately post contusion and the spinal cord irrigated with 0.9% saline. The computer connected to the impactor recorded the height, velocity at impact and cord displacement, providing graphical data. The cords of the animals and computer graphs were inspected immediately to assess for cord contusion, and accuracy of impaction. Animals that did not exhibit acceptable cord contusions, or poor graph analysis, were excluded.

### **Immediate Post-Injury Intervention.**

One hundred and twenty animals were randomized into four experimental groups (30 animals per group): 1) sham laminectomy without spinal cord injury, 2) spinal cord injury only, 3) spinal cord injury, intravenous saline, and 3 hours inhaled oxygen (FiO<sub>2</sub> 30%) at 5L/min in a chamber, 4) spinal cord injury, Oxycyte PFC emulsion 60% (5ml/kg), or saline (5ml/kg) flush and 3 hours of inhaled oxygen (FiO<sub>2</sub> 30%) at 5L/min in a chamber. The groups were further divided into six end points for sacrifice: Post-operative day (POD) 1, 4, 7, 14, 21, and 42. This schema provided five animals in each experimental treatment group at each of the end points (Table 1).

Appropriately injured animals were placed supine, and their jugular vein was accessed with a 25 gauge needle. The respective treatments were delivered through the jugular vein and injected

over a 2 minute period. All animals underwent paraspinal muscle approximation, skin closure using clips, and wound care with triple antibiotic gel.

All animals were placed on a homeothermic heating pad and recovered from anesthesia. All animals received post-operative resuscitative saline (0.9% sodium chloride) 5ml, prophylactic Gentamycin (5mg/kg) before surgery and for seven days thereafter and pain medication: Tylenol elixir (150mg/kg) in drinking water for the first 3 post-operative days, and Buprenorphine (0.5mg/kg) once pre-operatively, 12 hours after the initial dose and on an as needed basis. Manual bladder expression was performed every 12 hours post-operatively, until spontaneous micturition was re-established. Expressed urine was tested for signs of urinary tract infection (elevated pH or leukocytes). Additionally, if animals developed a urinary tract infection, Baytril (2.5mg/kg) was used q 12 hours, for five days. Animals were weighed weekly, and assessed for signs of distress (porphyria, self-mutilation). If they had lost 10% of their body weight, Nutri-Cal (1tsp daily feeds) supplementation was employed. Animals were housed two per cage and given free access to food and water.

### **Functional outcomes assessment**

Prior to surgery, all animals were handled in the open field maze once daily for 7 days prior to surgery. Locomotor function was graded using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale.<sup>37</sup> The BBB scale uses a range from zero (no hindlimb joint movements) to 21 (normal movements and coordinate gait). On the first post-operative day (POD1), and at POD: 4, 7, 14, 21 and 42, animals were placed in the open field and observed for four minutes. At each POD time point, five animals per group were tested. Two researchers blinded to the treatment group observed the animals in open-field testing. Hindlimb movement scores were averaged to obtain a single score for each animal per time point. The mean changes in BBB scores over time for the four groups were analyzed using repeated measures ANOVA with HSD post hoc t tests



between groups factor. The mean BBB scores were tallied by injured groups and plotted as a function of time after injury.

### **Incline plane test:**

Functional recovery of motor locomotion activity was also used to evaluate the animal ability to maintain their body position on an inclined plate.<sup>16</sup> Injured animals are prone to slip on steep inclined board. The test started at 15° climbing angle followed by incremental increase of 5° incline to a maximum of 45°. The maximum angle at which the animal could still maintain its position on the board for 10 sec was recorded for each animal. This test was performed 7, 14, 21, 35 and 42 days postoperative.

### **Post-Mortem Histological and Immunohistochemistry Analysis**

Three animals from each group provided tissue for histochemical analysis. Animals were sacrificed at 6 time points: Post-operative days 1, 4, 7, 14, 21 and 42. Animals were anesthetized with a lethal dose of pentobarbital (IP 100mg/kg), prior to exsanguination and underwent transcardiac perfusion with 250ml of 0.1M PBS, and 250ml of 4% paraformaldehyde. Perilesional spinal cord sections of 1cm were harvested, fixed and stored in 4% paraformaldehyde. Cord sections were cryoprotected in 0.1M PBS, pH 7.4 containing 30% sucrose overnight at 4°C prior to histological staining and immunohistochemistry. Spinal cord sections were blocked and embedded in optimal cutting temperature compound (Thermo Shandon, Pittsburgh, PA), and sectioned at 20µm, spanning the injury site and the perilesional area rostral and caudal to the lesion. Sections were mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were collected as sets of serial sections 100 µm between adjacent sections. To identify myelinated white matter and residual spared tissue, sectioned spinal cords were stained with Luxol Fast Blue (IHCWorld) and cresyl violet, respectively. Briefly spinal cord sections were rehydrated in graded alcohol by immersing for 5 minutes each in 100%, 95%, and then 70%

ethanol, followed by wash 2 time 5 minutes in 1X PBS at room temperature. After washing, slices were incubated in Luxol fast blue for overnight at 50 °C, and then treated with 0.05% Li<sub>2</sub>CO<sub>3</sub> followed by 70% ethanol. The sections were counterstained with Cresyl violet. Light microscopy at 25x magnification was used to visualize the sections, and images were obtained with an Olympus DP12 camera (Olympus America Inc. Center Valley, PA). An estimation of the percentage of spared tissue was calculated from tissue showing normal myelin appearance as described in detail<sup>55</sup>. Tissue containing septae and vacuoles were not considered spared tissues. The lesion epicenter of each injury was defined as the section with the least amount of spared white matter. The percentage of spared white matter and injured areas were calculated by dividing the total area of spared white matter and lesion area by the total spinal cord section, respectively. Mean values of percent spared white matter area were compared statistically using one-way ANOVA followed by Tukey HSD post hoc t tests *P* values of less than 0.05 were considered statistically significant. To determine neurons and oligodendrocytes cell death we used NeuroTACS II in situ detection kit (Trevigen, Gaithersburg, MD) based on terminal deoxynucleotidyl transferase enzyme TdT nick end labeling (TUNEL). The incorporated nucleotides are detected using a horseradish peroxidase system that catalyzes the conversion of diaminobenzidine (DAB) into a visible dark brown precipitate due to DNA fragmentation. Sections were hydrated and fixed as described for Luxol Blue staining followed by TUNEL staining suggested by the supplier. Negative controls sections were processed the same way but TdT enzyme was omitted from the labeling reaction. All negative controls did not have defined brown staining. For analysis of in situ apoptosis a Fluorescein or TMR-RED TUNEL detection kit was used according to the manufacturer's instructions (Roche diagnostic). For co-immunofluorescence to identify activated caspase 3 – positive and TUNEL – positive cells, slides were first TUNEL stained according to manufacturer's instructions and subsequently blocked and assayed with the activated caspase 3 antibody rabbit anti – cleaved caspase 3 rabbit

1:400 (Cell Signaling, MA.). For visualization Tetramethylrhodamine isothiocyanate (TRITC) secondary antibodies were used. TUNEL positive cells were counted in 10 randomly chosen fields per section. All kits were used according to the manufacturers' instructions.

## **Statistical analysis**

All data per group at each time point were summarized by basic descriptive statistics such as mean, standard deviation, and standard error of mean. The analysis of variance (ANOVA) method was used to compare the four groups in the amount of spared white matter and number of TUNEL positive cells at day 7 post-injury, respectively. The repeated measure analysis of variance was used to compare the four groups over time in BBB score, and TUNEL positive cells with four different locations per rat, respectively. Pair-wise comparisons of the groups were conducted in each outcome and the Tukey's procedure was used to adjust for the multiple comparisons to control the overall type I error of 5%. Data normality was checked in each outcome. SAS 9.2 was used for all analyses.

## **Results**

### **Intravenously administered Oxycyte reduced tissue damage and preserved myelin in the caudal peri-lesional area.**

Spinal cord injury is always accompanied by tissue ischemia and hypoxia. It was previously shown that following SCI, oxygen levels drop dramatically in the injured tissues followed by a series of destructive cellular processes which translate into cellular and neuronal death and loss of motor function.<sup>5,6,8,9</sup> The rationale for perfluorocarbon treatment is due to its high oxygen capacity and its ability to enhance parenchymal tissue oxygen levels after SCI.<sup>34</sup> We hypothesized that PFC can reverse neuronal damage by providing immediate high oxygen levels to injured tissue and neuron. Based on our previous work, the current study was designed to

examine how histopathology and motor function are affected by Oxycyte following spinal cord injury in rat-contusion model. We tested whether enhanced oxygen delivery with the assistance of Oxycyte, prevents tissue damage and preserves myelin following SCI. Intravenous Oxycyte (5ml/kg) was administrated to animals after SCI and compared to sham laminectomy, untreated SCI, and SCI plus saline and inhaled oxygen control groups. The cord sections rostral and caudal to the lesion epicenter were stained with Luxol Fast Blue and hematoxylin and eosin to determine white and grey matter damage and myelin preservation, as defined by the extent of preserved white matter fibers, and general tissue architecture, respectively. Analysis of myelin sparing (blue and green staining) revealed greater myelin and supportive tissue in all sections: epicenter, rostral, and caudal sections of PFC treated animals. As shown in the representative photomicrographs in figure 1, myelin and Nissl sparing, and tissue preservation was significantly better in Oxycyte-SCI group (Fig. 1. panel 4) as compared to the saline-SCI group (Fig. 1. panel 3) while spinal cord sections from the laminectomy control group did not show evidence of tissue or neuronal damage, demyelination, vacuoles or cavitation (Fig. 1. panel 1) in contrast to the SCI control which showed myelin loss, heterogeneous mixture of necrotic cells with shrunken cytoplasm and hypertrophic cells, (Fig. 1. panel 2; d,e,f). Histopathology staining of spinal cord sections from Oxycyte-injured group had significant reduction in tissue damage, showed significantly more myelin sparing (Fig. 1. j and k), and had better preserved Nissl and less vacuoles as shown in the H&E staining in (Fig. 1. l) when compared to saline animals which often showed significant tissue damage and less myelin (Fig. 1. g and h) as well significant spongy tissue, vacuoles and shrunken Nissl, typical of traumatic SCI shown in the H&E staining sections (Fig. 1. i). Furthermore, cresyl violet staining showed more preserved neurons in the Oxycyte-injured animal (Fig.1. P), while saline-injured groups showed distorted neuronal shape, rounding of cell bodies and the loss of neurons regular multipolar shape (Fig. 1. O).

## **White matter sparing and cystic lesions**

To assess the effects of Oxycyte on white matter and tissue damage Luxol fast blue-cresyl violet were used to stain spinal cord cross-sections of the different groups. The photomicrographs of Luxol fast blue-stained sections 24 hours post-contusion showed damage to the white and grey matter and loss of myelin in all spinal cord sections following SCI, with the most severe damage observed in the dorsal funiculus and dorsal horn while laminectomy sections have no evidence of tissue or myelin loss (Fig. 1A). The cystic lesions and tissue damage were extended at 6 weeks following spinal cord injury and cyst sizes were greater in the control-injured group than the Oxycyte-injured (Fig. 2B). The cord sections of Oxycyte-injured animals have reduced lesion size and better preserved myelin compared to the SCI and saline-group whereas no lesions or cavities were detectable in the sections of animals that sustained laminectomy. Also the myelin sheath collapsed into the area formerly occupied by the axon in the SCI control and saline-group, while the lesion is significantly less in the Oxycyte-group (Fig. 2B). Lesion size, and myelin destruction was assessed across all treatment and statistically analyzed at 1 and 6 weeks postoperative (Fig. 2C and 2E). Oxycyte treated animals showed significantly more preserved white matter compared to saline-injured animals ( $p=0.76$  in epicenter,  $p=0.31$  in rostral, and  $p=0.0015$  in caudal). Interestingly, Oxycyte had more profound white matter protection effect in sections caudal to the lesion epicenter than in rostral and epicenter consistent with the idea that Oxycyte can deliver oxygen to deep hypoxic tissues after SCI. The bar histogram (Fig. 2 C) compares the loss of tissue between the groups following traumatic injury. Luxol Blue staining of spinal cord sections caudal to the lesion epicenter showed that perfluorocarbons preserved approximately 50% of the baseline white matter at week 1 and 6 following SCI. The most significant statistical effects of Oxycyte on white matter preservation were observed in the caudal sections to the lesion epicenter which is consistent with the idea that Oxycyte can deliver

oxygen to deep hypoxic tissues after SCI. Myelin preservation in Oxycyte animals was statistically significant when compared to the SCI control, and to saline-injured animal with a p-value of 0.0004, and  $p=0.0015$ , respectively. In the epicenter and rostral sections, there was less significant difference in myelin preservation compared to the saline group as shown in the bar graph (Fig. 2 C). Furthermore, Oxycyte significantly reduced lesion size and tissue destruction in axial spinal cord sections at T10 at 6 weeks post-injury as shown by Luxol fast blue and Cresyl violet staining. Oxycyte treated animals had smaller intra-lesional cavity size while SCI controls and saline treated animals exhibited considerable demyelination and major loss of fibers and had larger lesions that often contained vacuoles typical of traumatic SCI (Fig. 2 B). Oxycyte-treated animals 42 days postoperative have significantly more spared white matter than saline-treated animals ( $P= 0.022$ ) as shown in figure 2E. Furthermore, Nissl-stained motor neuron in Oxycyte-treated animals were better preserved compared to saline-treated and injured groups shown in the photomicrographs of representative Nissl-stained sections (Fig. 2D).

### **In situ nick-end labeling and cellular death**

Spinal motor neuron and glia cells are particularly vulnerable to ischemia/hypoxia following the primary mechanical injury.<sup>38</sup> After acute spinal cord injury, apoptosis of neurons and oligodendrocytes occurs rapidly leading to a secondary pathologic cascade of tissue and neural injury.<sup>39-43</sup> We hypothesized that apoptotic cell death contributed to the axonal degeneration and white matter loss observed in Luxol Blue staining sections of injured animals (Fig. 1. And 2) and the significant tissue protection in the Oxycyte-injured animals is due in part to reduction of cellular death. To test this hypothesis we examined spinal cord section to detect apoptotic cell death using the TUNEL staining method. Apoptosis characterized by chromatin condensation, DNA fragmentation, nuclear shrinkage, and fragmentation of nuclear bodies (apoptotic bodies) was visualized by the in situ TUNEL technique as described in materials and methods. Animal groups were allowed to survive for 1, 4, 7, 14, 21 and 42 days post injury to analyze the amount

of cell death following SCI. The Oxycyte group had significantly fewer TUNEL positive cells (Fig. 3D); this was strikingly different to SCI-controls or to saline control animals, which had a greater degree of cell death ( $p=0.03$  and  $<0.0001$ , respectively) (Fig. 1 B, C). Figure 3A to D shows TUNEL-staining nuclei in gray and white matter of rostral spinal cord sections 7 days after SCI. Most TUNEL-staining neurons were located in dorsal horns and intermediate gray matter. Oxycyte treatment post-SCI significantly prevented ischemia-induced apoptosis in rostral (Fig. 3D) and caudal (Fig. 3H) sections at seven days post-injury. In addition, neuronal and oligodendrocytes cells in injured groups showed classical morphological features of apoptosis with HE staining, (Fig. 4A). We have also confirmed this in tissue stained sequentially with TUNEL and cleaved caspase 3, the TUNEL reactive cells co localized with cleaved caspase 3, indicating that the cells of injured animals were undergoing an apoptotic cell death (Fig. 4B).

### **TUNEL positive cell quantification**

For each animal two to three sections at 1, 4, 7, 14, 21 and 42 day post- injury were stained for TUNEL positive cells in the rostral and caudal sections, at day one, apoptotic cells were present in all sections from animals sustained injury and apoptosis levels increased progressively across time. The greatest level of apoptosis was seen at POD4 and 7, which correlates with the known secondary injury cascade and expected after SCI. As time passed, the degree of apoptosis decreased. The degree of apoptosis in the lesion epicenter across the six week post-injury course was compared across all groups. Oxycyte animals showed statistically significant less epicenter apoptosis across all six time points, as compared to the SCI control,  $p=0.0001$ . Oxycyte animals showed statistically significant less apoptosis versus saline animals across all six time points,  $p=0.0284$ . Saline animals showed statistically significant less apoptosis across six weeks compared to control SCI, with a  $p$ -value =  $0.0257$ , but less so than the Oxycyte group versus control SCI (Fig. 4C). Most sections of injured animal groups had significant number of cell death ( $p < 0.01$ ) ( $>40$  apoptotic cells  $\pm 2$ ), whereas the degree of cell death was significantly less

(15 TUNEL positive cells  $\pm$  1.02) in the Oxycyte group (Fig. 4C). TUNEL positive cells in the rostral sections, statistical significance was shown in Oxycyte treated animals versus control SCI;  $p=0.0001$ , and in Oxycyte animals versus saline animals,  $p=0.0072$ , but not between the saline versus SCI controls,  $p=0.1919$ . In caudal sections, statistical significance was shown in Oxycyte animals versus control SCI,  $p=0.0006$ , and in Oxycyte animals versus saline animals,  $p=0.0033$ , but not by saline versus SCI,  $p=0.7182$  (Fig. 4D). Most sections of injured animal groups had significant number of cell death ( $p < 0.01$ ) ( $>40$  apoptotic cells  $\pm$  2), whereas the degree of cell death was significantly less (15 TUNEL positive cells  $\pm$  1.02) in the Oxycyte group (Fig. 4C). There were no differences in the locations of TUNEL-staining neurons among injured animals. In this study, the number of TUNEL positive cells reached a peak at 1 week after SCI and decreased at 2 weeks post injury. Oxycyte treatment decreased the number of TUNEL positive cells at both 1 and 2 weeks after the spinal cord injury (Fig. 4C). In this study the apoptotic cell death in our model occurred mainly in the 1st week and that Oxycyte treatment significantly reduced the number of apoptotic cell death rostral and caudal to the lesion epicenter after spinal cord injury.

### **Motor function assessment.**

We next asked whether Oxycyte could enhance the recovery of hind limb locomotor function after SCI. We conducted an analysis of functional locomotor recovery after SCI using the BBB scale, as described in the materials and methods. All Laminectomy control group did not have functional deficits after 1 day postoperative. All animals developed complete paraplegia after SCI injury. Animals that achieved a score of 4 or higher at day 1 after injury were considered inadequate for the study; also animals with serious surgical complication were excluded from the experimental groups. The final groups for the BBB analysis were: SCI control ( $n = 28$ ), saline-injured ( $n = 20$ ), Oxycyte-injured control ( $n = 20$ ). BBB scores averaged were 0–1 on post-injury day 1, and all animal groups showed limited improvement at 7 days post-injury. Recovery



progressed slowly from week 2 to 6 as shown in Fig. 5A. Animals that received Oxycyte or intravenous saline post injury had better score at 21 days postoperative and they recovered faster than the injured control groups (Fig. 5A). The BBB scores of Oxycyte animals reached a plateau by 4 weeks with BBB scores similar to saline treated animals. BBB scores for Oxycyte and saline treated animals were  $14.6 \pm 1.05$  and  $14.2 \pm 1.92$  respectively at 6 weeks after SCI, which were significantly higher than those for the SCI control group;  $10.2 \pm 0.66$ . BBB scores of SCI controls were significantly lower than all groups at 2 weeks postoperative and remained significantly lower than all other groups during the remaining weeks (Fig. 5A). Although there was a trend for greater improvement of BBB scores in Oxycyte-treated animals at 2, 3 and 6 weeks after treatment, this improvement did not reach statistical significance over saline-injected animals. BBB scores improved over time in both Oxycyte and saline groups compared with SCI control animals, yet there was no statistically significant difference between the Oxycyte and SCI control animals,  $p= 2.4084$ ; Oxycyte and saline animals,  $p=-0.05017$ ; or saline versus SCI control animals,  $p=2.4586$ .

**Inclined plane test:** The results of the inclined plane test are presented in figure 5B. The inclined plane scores were analyzed by SigmaPlot 12.0 as shown in the lines graph (Fig. 5B). Oxycyte and saline group scores were higher than injured animal group, while the laminectomy group did not have any functional deficits or any disability postoperative. Results for the Oxycyte and saline group were similar.

## Discussion

Traumatic spinal cord injury results in the disruption of neural and vascular structures leading to ischemia/hypoxia and the initiation of the secondary pathogenic events that define the extent of functional recovery.<sup>8, 10, 44-46</sup> Indeed, many experimental SCI models have indicated that spinal cord traumatic injury is associated with long-lasting ischemia/hypoxia that parallels the force of

the insult and the severity of functional deficit.<sup>16, 47, 48</sup> To this date, no effective treatment is currently available for SCI, apart from steroids, which have modest protection, at best, and offer a host of potential risks. Since ischemia and the subsequent hypoxia translate into a worse neurological outcome and larger lesion size following SCI,<sup>49</sup> it is essential to provide oxygen to the hypoxic damaged tissues to reduce the ischemic injury in the hopes of preserving motor function. Enhanced oxygen levels in the hypoxic spinal cord tissue will correlate with better tissue and neuron preservation which may correct biochemical disturbances at the immediate and distal sites of spinal cord injury including metabolic enzymatic disturbance and inflammatory molecules, therefore reducing secondary spinal cord degeneration. We hypothesize that enhanced oxygen delivery via Oxycyte would preserve tissues and neurons after SCI since in animals and human studies PFCs have demonstrated improved outcomes following traumatic brain injury.<sup>29, 33</sup> In addition, we previously studied the effects of Oxycyte on tissue oxygenation post-SCI and demonstrated that perfusion with PFC significantly improved tissue oxygen level in the hypoxic tissues.<sup>34</sup> Based primarily on these findings, we tested the hypothesis that the addition of Oxycyte could be beneficial for decreasing ischemic injury and improving functional outcomes after SCI. This is the first time a third-generation PFC was used to evaluate its effects on tissue and neuron sparing after SCI. Enhancing oxygen level shortly after SCI, we observed at 7 and 42 days post-contusion that intravenous administration of Oxycyte significantly reduced apoptotic cell death and minimized tissue and neuronal damage in SCI animals compared to the saline treated group, which suggest a better functional recovery for Oxycyte-injured animals. These data are compatible with delayed deterioration of neurological function after spinal cord ischemic injury<sup>50</sup> and in agreement with the findings that tissue damage and white matter loss are major cause of sensory motor deficits that result from traumatic SCI.<sup>51, 52</sup> We can at least infer the involvement of apoptotic machinery in motor neuron death in which Oxycyte treatment reduced apoptosis and improved tissue and neuronal preservation. Our recent findings are in

agreement with our previous data showing that Oxycyte enhances oxygen level in the hypoxic tissue post-SCI<sup>34</sup> and consistent with the findings in brain injury that Oxycyte improves functional recovery post injury.<sup>29,33</sup> Thus, we have expected that locomotor function, as assessed by the BBB scale, would also be significantly affected because in the present study the degree of white matter loss and apoptotic cell death were significantly higher in the saline-injured group compared to the Oxycyte-injured group. Based on these findings, we hypothesized that functional recovery was likely to be better for the Oxycyte treated groups, since they had less cell death and better tissue and myelin preservation compared to saline groups, however motor recovery was similar for both groups. The reasons for the difference in histopathological and behavioral outcome in the present study may be a consequence of several variable factors. One explanation may lie on that most of the contusion damage were located in the dorsal lateral funiculus as well as the dorsal horn (Fig. 2A) which it contains mostly sensory functions related to fine touch, proprioceptive and vibration, while cell bodies of visceral and somatic motoneurons and descending fibers that compose the motor tracts were less damaged (Fig. 2B); the injury at T10 spinal cord level which the gray matter at this level may not contain specific group of neurons that can be selectively and precisely assessed by the BBB locomotor rating scale and most likely that BBB score alone is not designed to correspond exactly to the entire spectrum of possible functional outcomes. Other factor could be that the saline volume was large enough to produce significant hemodilution and perhaps hypertension which might have beneficial effect, as in the HHH therapy used in treating vasospasm after rupture of an intracranial aneurysm. Also behavioral testing is a complex endeavor with many contravening factors and could lack the specificity and sensitivity to detect small changes brought about by different treatment interventions or to identify particular neural substrates of motor behavior.<sup>53, 54</sup> Since most of the damage was observed in the dorsal horn, while the ventral horn had less damage thus motor function might not be greatly effected and it would be important in future

research to address other autonomic and sensory systems, particularly, bowel and bladder function and pain. Nevertheless BBB functional scores are not in conflict with the histopathological data because a significant reduction in cell death and better tissue and neuronal preservation known to correlate with better functional outcomes after SCI<sup>51,52</sup> thus, supporting a beneficial effect of Oxycyte in SCI and the continuation of its therapeutic evaluation. Regardless of the BBB scores, these data support a beneficial effect of Oxycyte in SCI and it will be necessary in future studies to determine the optimal of Oxycyte dose in animals with different contusion levels. Our study has revealed interesting insights into the role of PFCs in the reduction of neuronal cells and oligodendrocytes apoptosis and tissue damage after traumatic spinal cord injury, and suggests that more detailed investigations are needed to determine the optimal dose and schedule of administration depending upon the severity of the injury as well to define more mechanisms of action of Oxycyte. In summary, results of the current study have demonstrated that combined Oxycyte and O2 therapy has significant beneficial effects in protecting tissue and neurons and provide a strategy by which to limit secondary damage initiated by ischemia/hypoxia after SCI. Although our analysis was focused on Oxycyte as monotherapy for SCI, our overall findings raise the possibility that the administration of Oxycyte added as adjunctive therapy (such as approved FDA drug FTY720)<sup>55</sup> could enhance the possibility of optimal recovery of function following traumatic spinal cord injury.

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## **Author Disclosure Statement**

No competing financial interests exist.

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## **Figures Legends**

**FIG. 1. Oxycyte preserves myelin and white matter after SCI.** Light microscopic analysis Luxol fast blue-cresyl violet-stained sections 1mm caudal to the lesion epicenter 7 days after impaction (a to k). Panel **1**: animal group sustained laminectomy; **2**: animals sustained contusion; **3**: animal group sustained contusion and given saline (5ml/kg); **4**: animal group sustained contusion and given Oxycyte (5ml/kg). Representative micrographs (b,e,h,k) x 20 magnified area corresponding to x 2.5 micrograph of spinal cord area indicated by arrow head in sections (**a** to **j**). Note that myelin tracts (blue or green area) within the lesion are better preserved in Oxycyte treated animals while they are mostly lost in the saline group. Micrographs of Hematoxylin eosin-stained sections (c,f,i,l) correspond to the different levels of cellular damage

observed between the groups. The staining shows an extensive tissue and neuron damage, also hypertrophic cellular debris in contused untreated animals (f) while Oxycyte animals (l) have better preserved tissues and neurons (fewer vacuoles [V] and better preserved Nissl [N]) compared to tissular spongiosis and retracted Nissl in saline group (i). Cresyl violet staining (x 40) of motor neuronal cell bodies analyzed 7 days post-contusion shows large numbers of foamy macrophages and cellular debris (arrow head) in spinal cord sections of contused animal panel 2 (n) and injured Nissl in saline-injured (o) but better preserved neurons in Oxycyte-injured animals, while laminectomy alone showed normal motor neuronal cell bodies.

**FIG. 2. Oxycyte reduces lesion size and spares white matter following SCI.** Light microscopic analysis, Luxol fast blue-cresyl violet-staining of representative spinal cord cross-sections showing the different lesion size for the different treatment groups; saline-injured and Oxycyte-injured animals. The photomicrographs of Luxol fast blue-stained sections 24 hours post-contusion show damage to the white and grey matter and loss of myelin in all spinal cord sections following SCI, while laminectomy sections have no evidence of tissue or myelin loss. Note that most of the damage affects the gracile fasciculi and the dorsal horn, while ventral funiculus and horn have less damage (A). The cystic lesions and tissue damage was extended at 6 weeks following spinal cord injury and that cyst sizes were greater in the control-injured group than the Oxycyte-injured group. The cord sections of Oxycyte-injured animals have reduced lesion size (2B) and better myelin and Nissl preservation (2D) while SCI-control and SCI-saline groups have larger cysts (2B) and greater myelin loss and damage Nissl (2B), whereas in the section of animals that sustained laminectomy no lesions or cavities were detectable and no myelin or Nissl loss (Fig. 2B; 2D). Note that myelin sheath collapsed into the area formally occupied by the axon in the SCI control and saline group while it is significantly less in the Oxycyte group. Representative transverse spinal cord sections after T10 contusion injury were taken at 1 mm caudal to the lesion epicenter. Photomicrographs sections taken at x 2.5

magnification (A and B). Arrow heads in (B) indicated the enlarged area magnified x 40 in (D). E, Graph depicting area of white matter in spinal cord sections caudal to lesion epicenter of Oxycyte-treated and saline-treated animals as well SCI group that underwent spinal cord contusion at T9. Data represent means  $\pm$  se (n=3). Oxycyte-treated animals 42 days postoperative have significantly more spared white matter than saline-treated animals; there is a statistically significant difference between the saline and Oxycyte groups (P= 0.022, two-tailed Welch's t test comparison). Representative micrograph of spinal cord cross-sections 42 days after contusion is presented for each group above their statistical value.

**FIG. 3. Oxycyte reduces apoptotic cell death.** Representative photomicrographs (x 20) of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining illustrating apoptotic cell death after SCI. Spinal cords from rats after 7 days postoperative were processed and stained as described in the materials and methods section. Oxycyte administration suppressed SCI-induced apoptosis panels (H and D). The numbers of TUNEL-positive brown cells (arrowheads) are significantly higher in SCI and saline-injured groups (B and C). Oxycyte treatment protected spinal cord cell in both rostral (D) and caudal sections (H) from the lesion epicenter. Sections analyzed were from 2 mm rostral and caudal to the lesion epicenter. Laminectomy control showed only occasional labeled cells in the spinal cord sections (A and E).

**FIG. 4. Active caspase-3 co-localize with apoptotic cells.** High power photomicrograph (x 1000) of spinal cord sections TUNEL stained, showing characteristic apoptotic cell death (chromatin condensation and DNA fragmentation) in neuron (\*) and oligodendrocytes indicated by arrowhead (A). Co-immuno fluorescence of TUNEL with cleaved caspase 3 confirmed that TUNEL reactive cells were indeed apoptotic (B). Time course of apoptotic cell death following SCI presented in bar graph showing the number of TUNEL-positive nuclei 1 to 42 days post-injury (C). Oxycyte treated animals presented few apoptotic cell death compared to control-

injured and saline-injured animals (n = 3 cords in each group). Bar-graph presenting the number TUNEL positive cells in spinal cord sections 7 days post-injury. Apoptotic cells were calculated for rostral and caudal 1 mm to the lesion-epicenter (4D). Note that that administration of Oxycyte significantly reduced the apoptotic cell number in both rostral and caudal sections compared to the saline group. Asterisks indicate that means are significantly different from the saline-control group at the specified times after SCI (4D).

**FIG. 5. Oxycyte and saline treatment results in a trend toward motor enhanced function.**

Time course of functional recovery after spinal cord injury (SCI). (A) Graphical data represent mean open-field BBB scores of the vehicle-saline (n=5); oxycyte-treated animals (n=5) and contused animal (n=5) during the 6-week experimental period. All groups displayed the same partial recovery (BBB score=10) during the first 14 days, then diverged over the last 4 weeks, with Oxycyte and saline treatment resulting in a trend toward enhanced function. (B) The locomotor function was also tested by the inclined plane test on the same groups of animals as assessed in “A.” In most cases, motor function recovered more in the Oxycyte and saline treated groups than in the injured control groups. The Oxycyte and saline treated groups showed significant behavioral improvement (A) and significant ability to climb steeper inclined board compared with the injured untreated animals (B).

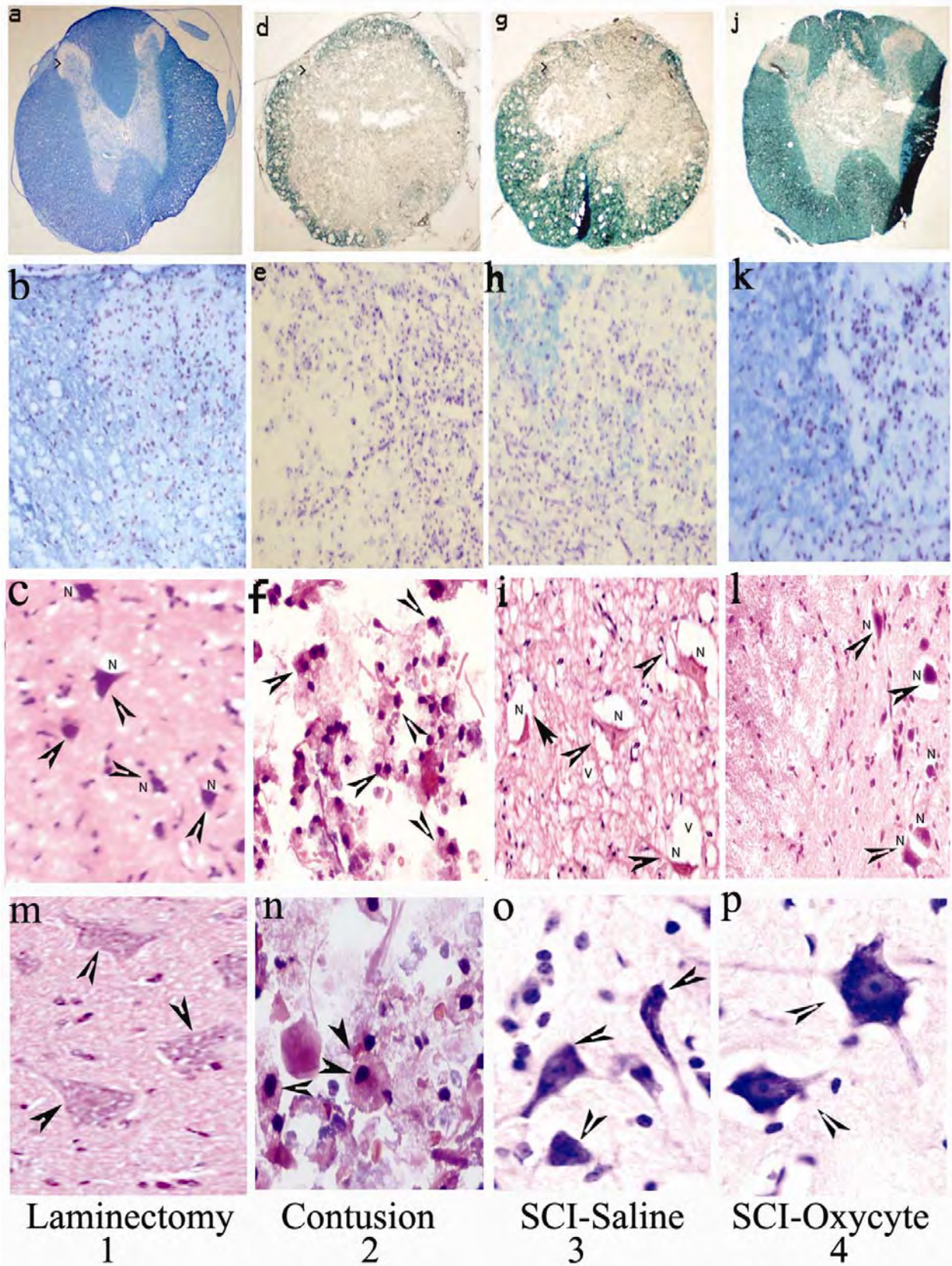


Figure 1.

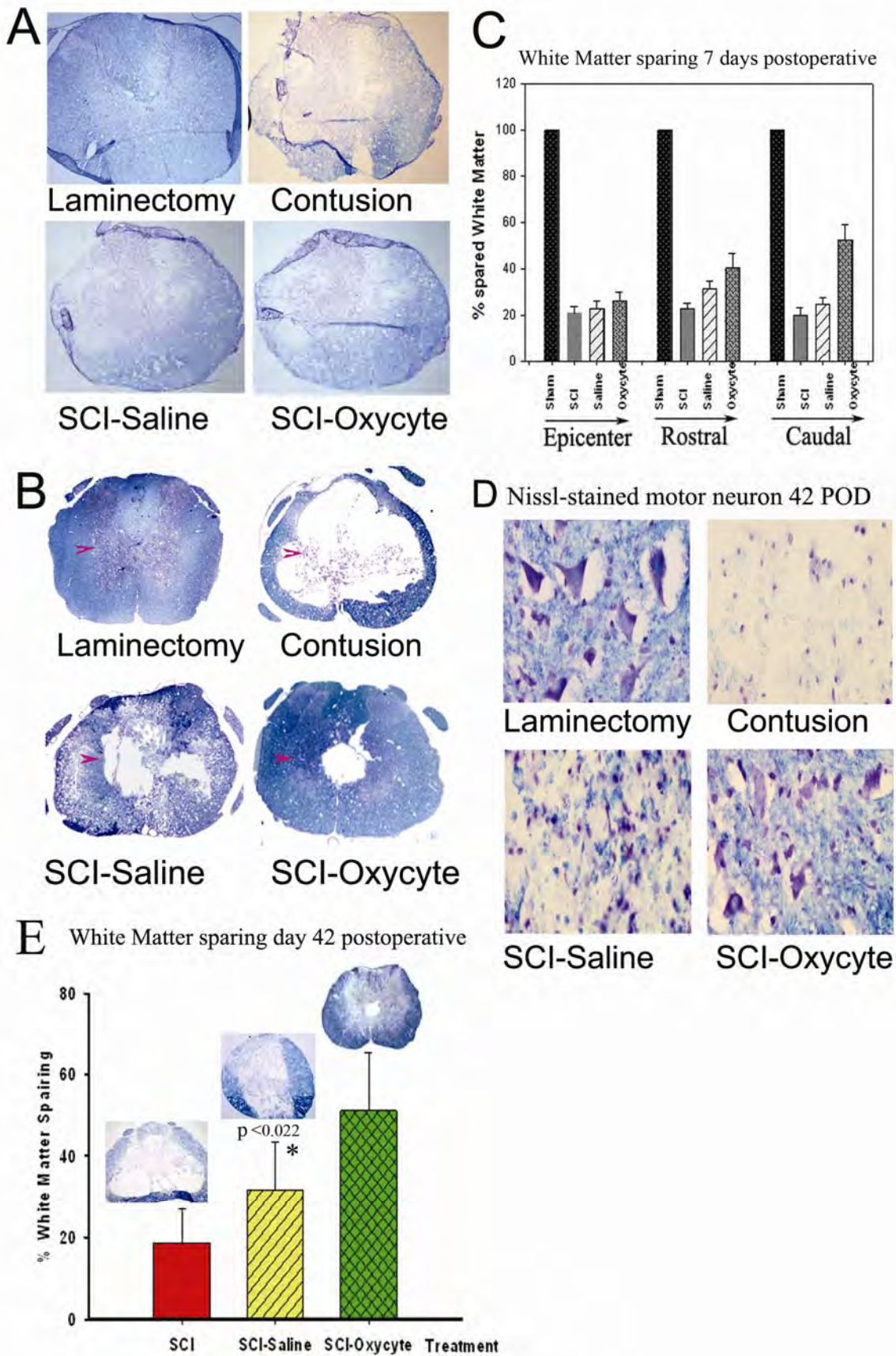
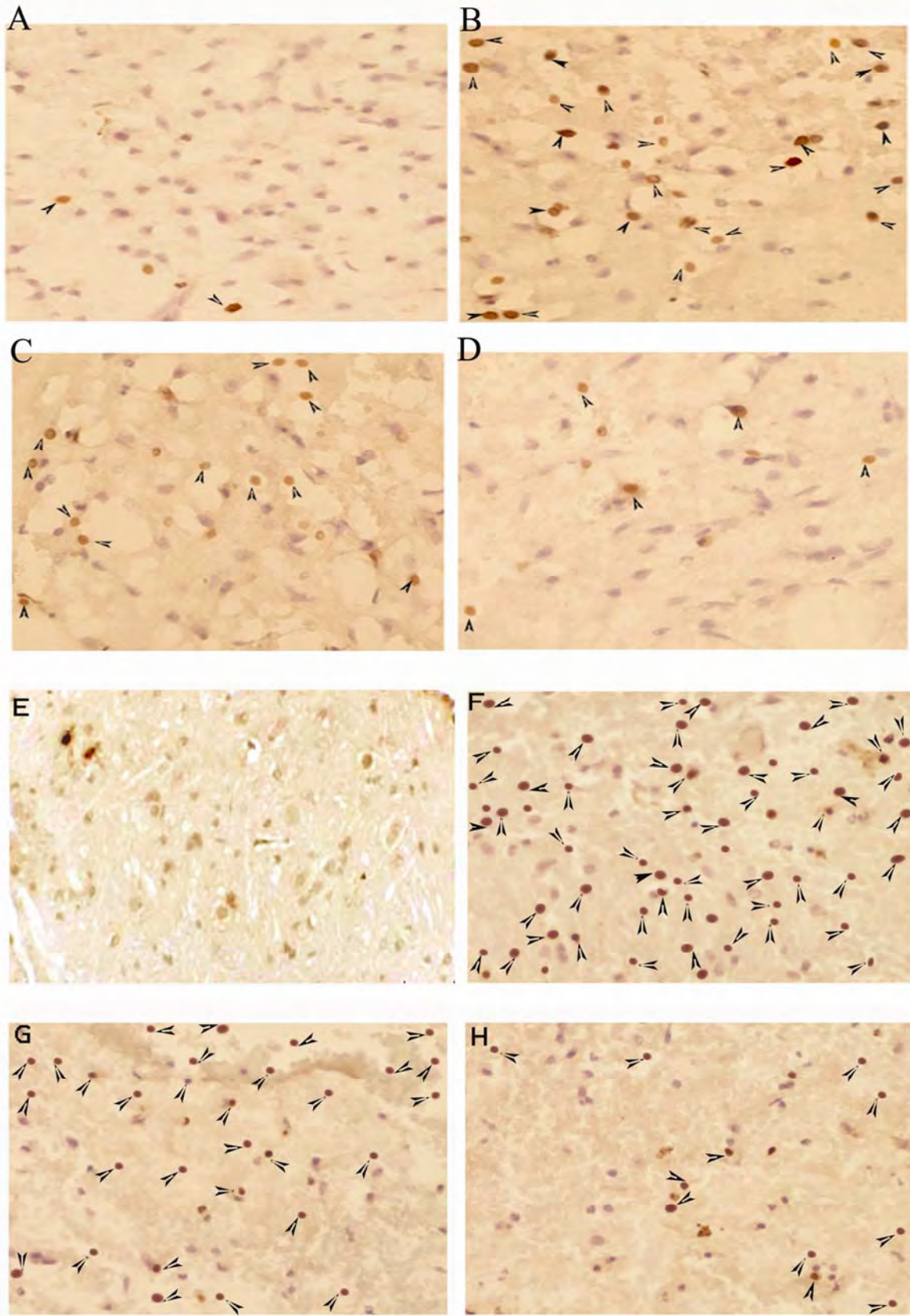
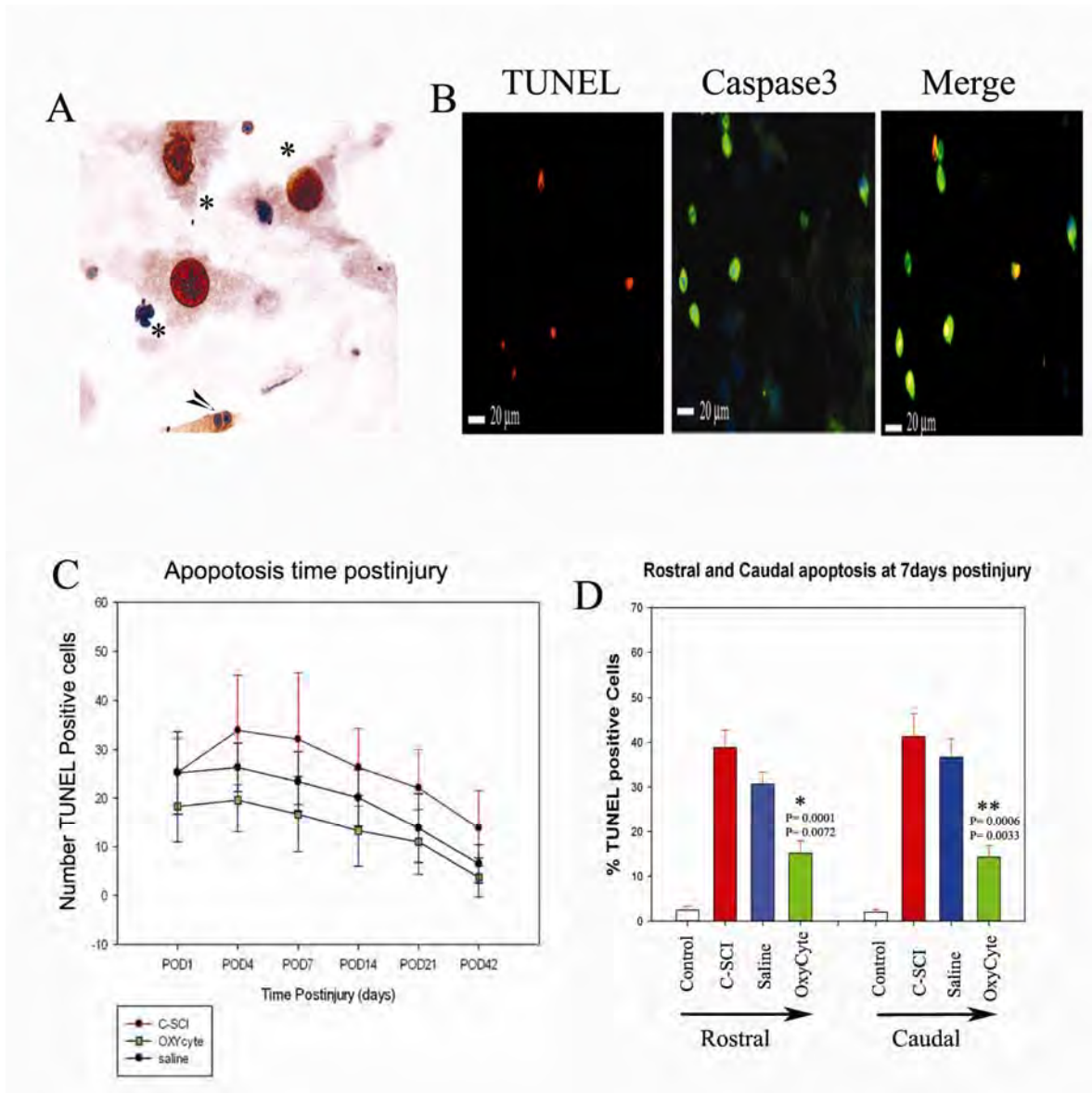


Figure 2.



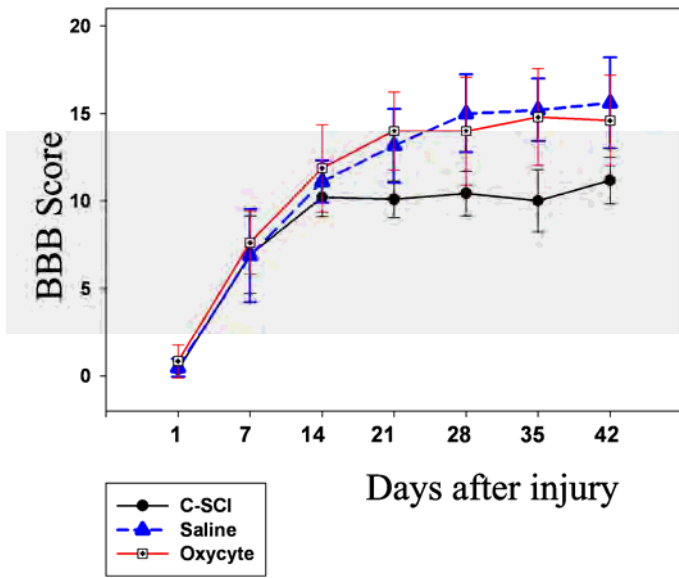


**Figure 3.**



**Figure 4.**

# A Locomotor Function Recovery



# B Inclined Panel Performance

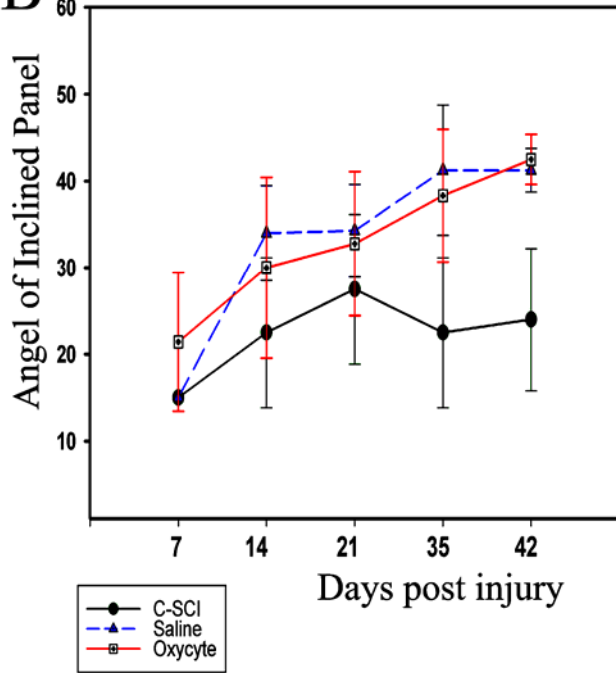


Figure 5.