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

Adly Yacoub,¹ Marygrace C. Hajec,¹ Richard Stanger,¹ Wen Wan,² Harold Young,¹ and Bruce E. Mathern¹

Abstract

Spinal cord injury (SCI) often results in irreversible and permanent neurological deficits and long-term disability. Vasospasm, hemorrhage, and loss of microvessels create an ischemic environment at the site of contusive or compressive SCI 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 postinjury 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 5 mL/kg of Oxycyte perfluorocarbon (60% emulsion) after SCI dramatically reduced destruction of spinal cord anatomy and resulted in a marked decrease of lesion area, less cell death, and greater white matter sparing at 7 and 42 days postinjury. Terminal deoxynucleotidyl transferase dUTP nick end labeling staining showed a significant reduced number of apoptotic cells in Oxycyte-treated animals, compared to the saline group. Collectively, these results demonstrate the potential neuroprotective effect of Oxycyte treatment after SCI, and its beneficial effects may be, in part, a result of reducing apoptotic cell death and tissue sparing. Further studies to determine the most efficacious Oxycyte dose and its mechanisms of protection are warranted.

Key words: apoptosis; ischemia; spinal cord injury

Introduction

SPINAL CORD INJURY (SCI) causes life-long disability and often permanent neurological deficits.¹ Despite advances in clinical research and rehabilitation strategies, there are still no effective treatment options to significantly improve functional recovery after SCI.^{2–4} SCI consists 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.^{5–10} 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.^{9,11–18} Among all of the secondary injuries, ischemia and hypoxia has been demonstrated as a focus of post-injury pathophysiological changes of acute SCI because it elicits the secondary injury complex cascade and increases the expression

of cell death markers as well as the activation of caspases that translate into cellular and neuronal death and worse neurological outcome.^{8,9,19–21} Therefore, improving spinal cord tissue oxygenation is a logical, 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.^{8,22} Decreased oxygen level in severe traumatic injuries appears to be implicated in poor functional outcome and death, and 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 cerebrospinal fluid, but parenchymal levels respond minimally^{23,24}; 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

¹Department of Neurosurgery, ²Department of Biostatistics, Virginia Commonwealth University, Richmond, Virginia.

minimize the ischemic injury. One promising candidate with good safety and tolerability is intravenous (i.v.) perfluorocarbon (PFC).^{25,26} PFCs are nontoxic oxygen carriers with an unrivaled capacity to dissolve gases, including oxygen. They are inert fluorinated hydrocarbons with highly efficient O₂ solubility and small particle size (<0.2 μ m). PFC's ability to increase O₂ delivery is based on its O₂ linear dissociation curve, making these molecule suitable for efficiently delivering oxygen to compromised ischemic neural tissues and can penetrate microcirculation of poor perfusion, as well as hold the promise of delivering therapy at the time of injury without the need of burdening logistics.²⁷ 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.^{28–32} Oxycyte, an injectable oxygen carrier, is a new generation of PFCs that have better property and fewer side effects, which was developed by Oxygen Biotherapeutics Inc. (Durham, NC). Oxycyte is a 60% emulsion of perfluoro-tert-butylcyclohexane with phospholipid emulsifiers. In several large and small animal models, PFCs were shown to preserve tissue and enhance motor function after traumatic brain injury (TBI),^{29,33} but its potential therapeutic effect in traumatic SCI 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 subsequent to SCI. To test this idea, we have previously shown that the pressure of oxygen levels dissolved in the blood in SCI dropped profoundly from 21.4 to 10.4 mmHg almost immediately postinjury, and all animals that received Oxycyte combined with 100% oxygen showed significant improvement, with a mean increase in oxygen levels of 23.3 mmHg.³⁴ These data confirmed that Oxycyte is an effective vehicle for oxygen delivery after SCI and can significantly increases tissue oxygen levels during the postinjury hypoxic phase.³⁴ 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 not been reported on. In the present study, we explored the role of Oxycyte in a rat model of SCI and hypothesized that a dose of PFC given i.v. after injury would significantly decrease neuronal and perineuronal cell death and apoptosis, as well as promote improved functional outcomes. Here, we report that Oxycyte reduces spinal cord damage and improves tissue sparing by directly reducing 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.

Methods

Animal model and surgical procedures

Long-Evans rats (Harlan Laboratories, Indianapolis, IL), 11–13 weeks old and weighing between 220 and 250 g, were used for SCI protocols. All housing as well as surgical and postoperative care procedures were performed in accord with the Department of Defense and the Virginia Commonwealth University (Richmond, VA) Institutional Animal Care and Use Committee. Animals were kept on a 12-h light/dark cycle and acclimatized for 1 week before surgery with feed, water, handling, and behavioral methods. Animals underwent anesthesia induction with 5% isoflurane and were maintained with 2.5% isoflurane during all surgical procedures. Depth of anesthesia was tested previously and throughout the surgical procedures by heart and respiration rate, as well as toe pinch. A rectal temperature probe was placed and the animal was

laid on a homeothermic blanket to maintain normothermia ($37.0 \pm 1^\circ\text{C}$). Animals received preincision 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 the aseptic technique, a left jugular vein exposure was completed, and then the animal was placed in a stereotactic frame. A dorsal mid-line incision was made over the mid-thoracic spine, using the T5 prominence as a guide. Paraspinal muscles were carefully dissected off the spinous process, as well as lamina. A standard T 9/10 laminectomy was performed using microrongeurs to make the spinal cord and exposed dura ready for cord contusion. The animal was then placed on the Multicenter Animal Spinal Cord Injury Study (MASCIS) II-NYU (New York University) Impactor platform^{35,36} 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 (10 g) was dropped from a height of 25 mm (range, 6.25–50.0), producing a moderate-to-severe cord contusion/SCI, resulting in hindlimb paralysis and neurogenic bladder. The impactor rod was removed immediately postcontusion, and the spinal cord was 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, as well as accuracy of impaction. Animals that did not exhibit acceptable cord contusions, or poor graph analysis, were excluded.

Immediate postinjury intervention

One hundred and twenty animals were randomized into four experimental groups (30 animals per group): 1) sham laminectomy without SCI; 2) SCI only; 3) SCI, i.v. saline, and 3 h of inhaled oxygen (FiO₂ 30%) at 5 L/min in a chamber; and 4) SCI, Oxycyte PFC emulsion 60% (5 mL/kg), or saline (5 mL/kg) flush and 3 h of inhaled oxygen (FiO₂ 30%) at 5 L/min in a chamber. The groups were further divided into six end-points for sacrifice: postoperative day (POD) 1; 4; 7; 14; 21; and 42. This schema provided 5 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. Respective treatments were delivered through the jugular vein and injected over a 2-min 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 postoperative resuscitative saline (0.9% sodium chloride, 5 mL), prophylactic

TABLE 1. DESCRIPTION OF EXPERIMENTAL GROUPS

Experimental group	BBB score	Histology	TUNEL staining
SCI contusion control	n = 5	n = 3	n = 3
SCI + saline	n = 5	n = 3	n = 3
SCI + oxycyte	n = 5	n = 3	n = 3
Laminectomy	n = 5	n = 3	n = 3

Description of experimental groups: 120 animals were divided into four experimental groups (30 per group): (1) sham; (2) untreated-SCI; (3) SCI + saline + oxygen; and (4) SCI + Oxycyte + oxygen, over six end temporal end-points: (postoperative days 1, 4, 7, 14, 21, and 42). Five animals served for BBB analysis at each respective time point in each experimental group. Histochemical analysis was completed on 3 animals from each group.

SCI, spinal cord injury; BBB, Basso-Beattie-Bresnahan scale; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

gentamycin (5 mg/kg) before surgery and for 7 days thereafter, and pain medication: Tylenol elixir (150 mg/kg) in drinking water for the first 3 PODs and buprenorphine (0.5 mg/kg) once preoperatively, 12 h after the initial dose, and on an as-needed basis. Manual bladder expression was performed every 12 h postoperatively, until spontaneous micturition was reestablished. Expressed urine was tested for signs of urinary tract infection (UTI; elevated pH or leukocytes). Additionally, if animals developed a UTI, Baytril (2.5 mg/kg) was used q 12 h, for 5 days. Animals were weighed weekly and assessed for signs of distress (porphyria or self-mutilation). If they had lost 10% of their body weight, Nutri-Cal (1-teaspoon daily feeds) supplementation was employed. Animals were housed 2 per cage and given free access to food and water.

Functional outcomes assessment

Before surgery, all animals were handled in the open-field maze once-daily for 7 days preceding surgery. Locomotor function was graded using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale.³⁷ The BBB scale uses a range from zero (no hindlimb joint movements) to 21 (normal movements and coordinate gait). On the first postoperative day (POD1), and at PODs 4, 7, 14, 21, and 42, animals were placed in the open field and observed for 4 min. At each POD time point, 5 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 analysis of variance (ANOVA) with the honestly significant difference (HSD) post-hoc *t*-tests between-groups factor. 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 animals' ability to maintain their body position on an inclined plate.¹⁶ Injured animals are prone to slip on a steep inclined board. The test started at a 15-degree climbing angle followed by an incremental increase of a 5-degree incline to a maximum of 45 degrees. 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 postoperatively.

Postmortem histological and immunohistochemistry analysis

Three animals from each group provided tissue for histochemical analysis. Animals were sacrificed at six time points: PODs 1, 4, 7, 14, 21, and 42. Animals were anesthetized with a lethal dose of pentobarbital (100 mg/kg, intraperitoneally) before exsanguination and underwent transcardiac perfusion with 250 mL of 0.1 M of phosphate-buffered saline (PBS) and 250 mL of 4% paraformaldehyde (PFA). Perilesional spinal cord sections of 1 cm were harvested, fixed, and stored in 4% PFA. Cord sections were cryoprotected in 0.1 M of PBS (pH 7.4) containing 30% sucrose overnight at 4°C before histological staining and immunohistochemistry. Spinal cord sections were blocked and embedded in optimal cutting temperature compound (Thermo Shandon, Pittsburgh, PA) and sectioned at 20 μ m, 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 μ m between adjacent sections. To identify myelinated white matter (WM) and residual spared tissue, sectioned spinal cords were stained with Luxol Fast Blue (IHC World, LLC, Woodstock, MD) and cresyl violet, respectively. Briefly, spinal cord sections were

rehydrated in graded alcohol by immersing for 5 min each in 100, 95, and then 70% ethanol, followed by washing two times for 5 min in 1 $\times$ PBS at room temperature. After washing, slices were incubated in Luxol Fast Blue overnight at 50°C and then treated with 0.05% Li₂CO₃ followed by 70% ethanol. Sections were counterstained with cresyl violet. Light microscopy at 25 $\times$ 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 elsewhere.⁵⁵ Tissues 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 WM. The percentage of spared WM and injured areas was calculated by dividing the total area of spared WM and lesion area by the total spinal cord section, respectively. Mean values of percent spared WM area were compared statistically using one-way ANOVA followed by Tukey's HSD post-hoc *t*-tests. *p* values of less than 0.05 were considered statistically significant. To determine neurons and oligodendrocyte cell death, we used the 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 into a visible dark brown precipitate resulting from DNA fragmentation. Sections were hydrated and fixed, as described by Luxol Blue staining, followed by TUNEL staining, as 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 Diagnostics, Indianapolis, IN). For coimmunofluorescence to identify activated caspase 3- and TUNEL-positive cells, slides were first TUNEL stained according to manufacturer instructions and subsequently blocked and assayed with the activated caspase 3 antibody (Ab), rabbit anti-cleaved caspase 3 rabbit (1:400; Cell Signaling Technology, Inc., Danvers, MA). For visualization, tetramethylrhodamine isothiocyanate secondary Abs were used. TUNEL-positive cells were counted in 10 randomly chosen fields per section. All kits were used according to the manufacturer's 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 ANOVA method was used to compare the four groups in the amount of spared WM and number of TUNEL-positive cells at day 7 postinjury, respectively. Repeated-measure ANOVA 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 groups were conducted in each outcome, and 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 statistical software (9.2; SAS Institute Inc., Cary, NC) was used for all analyses.

Results

Intravenously administered Oxycyte reduced tissue damage and preserved myelin in the caudal perilesional area

SCI is always accompanied by tissue ischemia and hypoxia. It was previously shown that after SCI, oxygen levels drop dramatically in the injured tissues, followed by a series of destructive cellular processes that translate into cellular and neuronal death and

loss of motor function.^{5,6,8,9} The rationale for PFC treatment is because of its high oxygen capacity and ability to enhance parenchymal tissue oxygen levels after SCI.³⁴ We hypothesized that PFC could reverse neuronal damage by providing immediate high oxygen levels to injured tissue and neurons. Based on our previous work, the current study was designed to examine how histopathology and motor function are affected by Oxycyte after SCI in a rat-contusion model. We tested whether enhanced oxygen delivery, with the assistance of Oxycyte, prevents tissue damage and preserves myelin after SCI. I.v. Oxycyte (5 mL/kg) was administered to animals after SCI and compared to sham laminectomy, untreated SCI, and SCI plus saline and inhaled oxygen control groups. Cord sections rostral and caudal to the lesion epicenter were stained with Luxol Fast Blue and hematoxylin and eosin (H&E) to determine WM and gray matter (GM) damage and myelin preservation, as defined by the extent of preserved WM 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, as well as tissue preservation, was significantly better in the Oxycyte-SCI group (Fig. 1, panel 4), as compared to the saline-SCI group (Fig. 1, panel 3), whereas 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–f). Histopathology staining of spinal cord sections from the Oxycyte-injured group had a 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-treated 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). Further, cresyl violet staining showed more-preserved neurons in Oxycyte-injured animals (Fig. 1, p), whereas saline-injured groups showed distorted neuronal shape, rounding of cell bodies, and loss of neurons of regular multi-polar shape (Fig. 1, o).

White matter sparing and cystic lesions

To assess effects of Oxycyte on WM 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 h postcontusion showed damage to the WM and

GM and loss of myelin in all spinal cord sections after SCI, with the most severe damage observed in the dorsal funiculus and dorsal horn, whereas the laminectomy sections had no evidence of tissue or myelin loss (Fig. 2A). Cystic lesions and tissue damage were extended at 6 weeks after SCI, and cyst sizes were greater in the control-injured group than the Oxycyte-injured group (Fig. 2B). Cord sections of Oxycyte-injured animals had reduced lesion size and better-preserved myelin, compared to the SCI and saline groups, 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 groups, whereas the lesion was significantly less in the Oxycyte group (Fig. 2B). Lesion size and myelin destruction were assessed across all treatments and statistically analyzed at 1 and 6 weeks postoperatively (Fig. 2C,E). Oxycyte-treated animals showed significantly more preserved WM, 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 WM 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. 2C) compares the loss of tissue between the groups after traumatic injury. Luxol Blue staining of spinal cord sections caudal to the lesion epicenter showed that PFCs preserved approximately 50% of baseline WM at weeks 1 and 6 after SCI. The most significant statistical effects of Oxycyte on WM 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-treated animals was statistically significant, when compared to the SCI control and saline-injured animals ($p=0.0004$ 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. 2C). Further, Oxycyte significantly reduced lesion size and tissue destruction in axial spinal cord sections at T10 at 6 weeks postinjury, as shown by Luxol Fast Blue and cresyl violet staining. Oxycyte-treated animals had smaller intralesional cavity size, whereas 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. 2B). Oxycyte-treated animals 42 days postoperatively had significantly more spared WM than saline-treated animals ($p=0.022$; Fig. 2E). Further, Nissl-stained motor neurons 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).

FIG. 1. Oxycyte preserves myelin and white matter after SCI. Light microscopic analysis Luxol Fast Blue/cresyl violet-stained sections 1 mm caudal to the lesion epicenter 7 days after impactation (A–K). Panel 1: animal group sustained laminectomy; 2: animals sustained contusion; 3: animal group sustained contusion and given saline (5 mL/kg); 4: animal group sustained contusion and given Oxycyte (5 mL/kg). Representative micrographs (B, E, H, and K) 20× magnified area corresponding to 2.5× micrograph of spinal cord area indicated by arrowhead in sections (A–J). Note that myelin tracts (blue or green area) within the lesion are better preserved in Oxycyte-treated animals, whereas they are mostly lost in the saline group. Micrographs of hematoxylin and eosin-stained sections (C, F, I, and L) correspond to the different levels of cellular damage observed between the groups. Staining shows an extensive tissue and neuron damage, as well as hypertrophic cellular debris in contused untreated animals (F), whereas 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 the saline group (I). Cresyl violet staining (40×) of motor neuronal cell bodies analyzed 7 days postcontusion 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 animals (O), but better-preserved neurons in Oxycyte-injured animals, whereas laminectomy alone showed normal motor neuronal cell bodies. SCI, spinal cord injury. Color image is available online at www.liebertpub.com/neu

In situ nick end labeling and cellular death

Spinal motor neuron and glia cells are particularly vulnerable to ischemia and hypoxia subsequent to the primary mechanical injury.³⁸ After acute SCI, apoptosis of neurons and oligodendrocytes occurs

rapidly, leading to a secondary pathologic cascade of tissue and neural injury.^{39–43} We hypothesized that apoptotic cell death contributed to the axonal degeneration and WM loss observed in Luxol Blue staining sections of injured animals (Figs. 1 and 2), and the significant tissue protection in Oxycyte-injured animals was, in

part, the result of reduction of cellular death. To test this hypothesis, we examined spinal cord sections 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 above. Animal groups were allowed to survive for 1, 4, 7, 14, 21, and 42 days postinjury to analyze the amount of cell death after 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. 1B,C). Figure 3A–D shows TUNEL staining nuclei in GM and WM of rostral spinal cord sections 7 days after SCI. Most TUNEL staining neurons were located in dorsal horns and intermediate GM. Oxycyte treatment post-SCI significantly prevented ischemia-induced apoptosis in rostral (Fig. 3D) and caudal (Fig. 3H) sections at 7 days postinjury. In addition, neuronal and oligodendrocyte cells in injured groups showed classical morphological features of apoptosis with H&E staining (Fig. 4A). We have also confirmed this in tissue stained sequentially with TUNEL and cleaved caspase 3; TUNEL-reactive cells colocalized with cleaved caspase 3, indicating that 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 days postinjury were stained for TUNEL-positive cells in the rostral and caudal sections; at day 1, apoptotic cells were present in all sections from animals that sustained injury and apoptosis levels increased progressively across time. The greatest level of apoptosis was observed at POD4 and 7, which correlates with the known secondary injury cascade and what is expected after SCI. As time passed, degree of apoptosis decreased. Degree of apoptosis in the lesion epicenter across the 6-week postinjury course was compared across all groups. Oxycyte animals showed statistically significantly 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 significantly less apoptosis across 6 weeks, compared to control SCI ($p=0.0257$), but less so than the Oxycyte group versus control SCI (Fig. 4C). Most sections of injured animal groups had signif-

icant numbers of cell death ($p<0.01$; >40 apoptotic cells ± 2), whereas degree of cell death was significantly less (15 TUNEL-positive cells ± 1.02) in the Oxycyte group (Fig. 4C). In TUNEL-positive cells in rostral sections, statistical significance was shown in Oxycyte-treated animals versus control SCI ($p=0.0001$) and in Oxycyte 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 numbers of cell death ($p<0.01$; >40 apoptotic cells ± 2), whereas degree of cell death was significantly less (15 TUNEL-positive cells ± 1.02) in the Oxycyte group (Fig. 4C). There were no differences in 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 postinjury. Oxycyte treatment decreased the number of TUNEL-positive cells at both 1 and 2 weeks after SCI (Fig. 4C). In this study, apoptotic cell death in our model occurred mainly in the first week, and Oxycyte treatment significantly reduced the numbers of apoptotic cell death rostral and caudal to the lesion epicenter after SCI.

Motor function assessment

We next asked whether Oxycyte could enhance the recovery of hindlimb locomotor function after SCI. We conducted an analysis of functional locomotor recovery after SCI using the BBB scale, as described above. All animals in the laminectomy control group had no functional deficits after 1 day postoperatively. 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 ($n=20$), and Oxycyte-injured control ($n=20$). BBB scores averaged 0–1 on postinjury day 1, and all animal groups showed limited improvement at 7 days postinjury. Recovery progressed slowly from weeks 2–6, as shown in Figure 5A. Animals that received Oxycyte or i.v. saline postinjury had a better score at 21 days postoperatively and recovered faster than injured control groups (Fig. 5A). BBB scores of Oxycyte animals reached a plateau by 4 weeks, with BBB scores similar to saline-treated animals. BBB

FIG. 2. Oxycyte reduces lesion size and spares white matter after 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- and Oxycyte-injured animals). The photomicrographs of Luxol Fast Blue-stained sections 24 h postcontusion show damage to the white and gray matter and loss of myelin in all spinal cord sections after SCI, whereas laminectomy sections have no evidence of tissue or myelin loss. Note that most of the damage affects the gracile fasciculi and the dorsal horn, whereas the ventral funiculus and horn have less damage (A). The cystic lesions and tissue damage were extended at 6 weeks after SCI and cyst sizes were greater in the control-injured group than the Oxycyte-injured group. Cord sections of Oxycyte-injured animals have reduced lesion size (B) and better myelin and Nissl preservation (D), whereas SCI-control and SCI-saline groups have larger cysts (B) and greater myelin loss and damage Nissl (D), whereas in the section of animals that sustained laminectomy, no lesions or cavities were detectable and no myelin or Nissl loss (B and D). Note that myelin sheath collapsed into the area formally occupied by the axon in the SCI-control and saline groups, whereas it was 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 $2.5\times$ magnification (A and B). Arrow heads in (B) indicated the enlarged area magnified $40\times$ in (D). (C) A graphic summary of the mean percentages of spared white matter rostral and caudal to lesion epicenter 7 days postinjury. (E) Graph depicting area of white matter in spinal cord sections caudal to lesion epicenter of Oxycyte- and saline-treated animals as well the SCI group that underwent spinal cord contusion at T9. Data represent means $\pm$ standard error ($n=3$). Oxycyte-treated animals 42 days postoperatively had significantly more spared white matter than saline-treated animals; there was a statistically significant difference between the saline and Oxycyte groups ($p=0.022$, Welch's two-tailed *t*-test comparison). Representative micrograph of spinal cord cross-sections 42 days after contusion is presented for each group above their statistical value. SCI, spinal cord injury; POD, postoperative day. Color image is available online at www.liebertpub.com/neu

FIG. 3. Oxycyte reduces apoptotic cell death. Representative photomicrographs (20 $\times$) 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 postoperatively were processed and stained as described in the Methods section. Oxycyte administration suppressed SCI-induced apoptosis rostral (**D**) and caudal from the lesion epicenter (**H**). The numbers of TUNEL-positive cells (arrowheads) are significantly higher in SCI (**B** and **F**) and saline-injured groups (**C** and **G**). Oxycyte treatment protected spinal cord cells in both rostral (**D**) and caudal sections (**H**) from the lesion epicenter. Sections analyzed were from 2 mm rostral (**A–D**) and caudal to the lesion epicenter (**E–H**). Laminectomy control showed only occasional labeled cells in the spinal cord sections (**A** and **E**). SCI, spinal cord injury.

FIG. 4. Active caspase 3 colocalize with apoptotic cells. High-power photomicrograph (1000 $\times$) of spinal cord sections that were TUNEL stained, showing characteristic apoptotic cell death (chromatin condensation and DNA fragmentation) in neuron (*) and oligodendrocytes indicated by arrowhead (A). Coimmunofluorescence of TUNEL with cleaved caspase 3 confirmed that TUNEL-reactive cells were indeed apoptotic (B). Time course of apoptotic cell death after SCI presented in bar graph showing the number of TUNEL-positive nuclei 1–42 days postinjury (C) Oxyocyte-treated animals presented few apoptotic cell deaths, compared to control- and saline-injured animals ($n = 3$ cords in each group). Bar graph presenting the number of TUNEL-positive cells in spinal cord sections 7 days postinjury. Apoptotic cells were calculated for rostral and caudal 1 mm to the lesion epicenter (D). Note that administration of Oxyocyte significantly reduced 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 (D). TUNEL, deoxynucleotidyl transferase enzyme TdT nick end labeling; SCI, spinal cord injury.

scores for Oxyocyte and saline-treated animals were 14.6 ± 1.05 and 14.2 ± 1.92 , respectively, at 6 weeks after SCI, which were significantly higher than those for the SCI control group; 10.2 ± 0.66 . BBB scores of SCI controls were significantly lower than all groups at 2 weeks postoperatively 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 Oxyocyte-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 Oxyocyte and saline groups, compared to SCI control animals; yet, there was no statistically significant difference between the Oxyocyte and SCI control animals ($p = 2.4084$), Oxyocyte 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). Oxyocyte and saline group scores were higher than the injured animal group, whereas the laminectomy group did not have any functional deficits or any disability postoperatively. Results for the Oxyocyte and saline groups were similar.

Discussion

Traumatic SCI results in the disruption of neural and vascular structures, leading to ischemia and hypoxia and the initiation of the secondary pathogenic events that define the extent of functional

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 ($n=5$) and oxycyte-treated animals ($n=5$) and contused animals ($n=5$) 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) 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 a steeper inclined board, compared to injured untreated animals (B). BBB, Basso-Beattie-Bresnahan scale.

recovery.^{8,10,44–46} Indeed, many experimental SCI models have indicated that spinal cord traumatic injury is associated with long-lasting ischemia and hypoxia that parallels the force of the insult and the severity of functional deficit.^{16,47,48} To 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. Because ischemia and subsequent hypoxia translate into a worse neurological outcome and larger lesion size after SCI,⁴⁹ it is essential to provide oxygen to the hypoxic-damaged tissues to reduce ischemic injury in the hope of preserving motor function. Enhanced oxygen levels in hypoxic spinal cord tissue will correlate with better tissue and neuron preservation, which may correct biochemical disturbances at the immediate and distal sites of SCI, including metabolic enzymatic disturbance and inflammatory molecules, therefore reducing secondary spinal cord degeneration. We hypothesize that enhanced oxygen delivery by Oxycyte would preserve tissues and neurons after SCI, because in animal and human studies, PFCs have demonstrated improved outcomes after TBI.^{29,33} 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 hypoxic tissues.³⁴ 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 that 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 postcontusion, that i.v. administration of Oxycyte significantly reduced apoptotic cell death and minimized tissue and neuronal damage in SCI animals, compared to the saline-treated group, which suggests a better functional recovery for Oxycyte-injured animals. These data are compatible with delayed deterior-

ation of neurological function after spinal cord ischemic injury⁵⁰ and are in agreement with the findings that tissue damage and WM loss are a major cause of sensory motor deficits that result from traumatic SCI.^{51,52} 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 hypoxic tissue post-SCI³⁴ and consistent with the findings in brain injury that Oxycyte improves functional recovery postinjury.^{29,33} Thus, we have expected that locomotor function, as assessed by the BBB scale, would also be significantly affected, because in the present study, degree of WM 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, because 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 in that most of the contusion damage were located in the dorsal lateral funiculus as well as the dorsal horn (Fig. 2A), which contains mostly sensory functions related to fine touch, proprioceptiveness, and vibration, whereas cell bodies of visceral and somatic motor neurons and descending fibers that compose the motor tracts were less damaged (Fig. 2B); for the injury at the T10 spinal cord level, in which the GM at this level may not contain a specific group of neurons that can be selectively and precisely assessed by the BBB locomotor rating scale, most likely, the BBB score alone is not designed to correspond exactly to the entire spectrum of possible functional outcomes. Other factors could be

that saline volume was large enough to produce significant hemodilution and perhaps hypertension, which might have a beneficial effect, as in hypervolemia, hypertension, and hemodilution (triple-H) 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 identify particular neural substrates of motor behavior.^{53,54} Because most of the damage was observed in the dorsal horn, whereas the ventral horn had less damage and thus motor function might not be greatly effected, 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 are known to correlate with better functional outcomes after SCI,^{51,52} 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 oligodendrocyte apoptosis and tissue damage after traumatic SCI 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 and 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 (e.g., U.S. Food and Drug Administration–approved drug FTY720)⁵⁵ could enhance the possibility of optimal recovery of function after traumatic SCI.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Bruce E. Mathern, MD

Department of Neurosurgery

VCU Medical Center

Virginia Commonwealth University

417 North 11th Street, 6th Floor

PO Box 980631

Richmond, VA 23298

E-mail: BMathern@mcvh-vcu.edu