ESTABLISHMENT AND CHARACTERIZATION OF AN ANIMAL MODEL OF

DISTRACTION SPINAL CORD INJURY

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2013

ACKNOWLEDGEMENTS

When I reflect upon my graduate career, I am overwhelmed with thankfulness for God's grace and provision. First and foremost, I would like to express my deepest gratitude to my husband, Brad Bell, for his truly unconditional love and support. He has been a constant source of wisdom and encouragement throughout this challenging journey. I am also especially grateful for my parents, Kay and Bill Stearns, who believed in my academic abilities long before I did and have provided unwavering support throughout my life.

Professionally, I would like to thank my research advisor, Mario Romero-Ortega, for giving me the opportunity to join his team and pursue this research. His support throughout my graduate career has been invaluable. I would also like to thank my committee members, Daniel Sucato, Yuan Bo Peng, Young-tae Kim, and Kytai Nguyen, for their guidance in the preparation of this dissertation.

I would also like to express deep gratitude to Jennifer Seifert for her invaluable mentorship and her words of advice and encouragement throughout my graduate career.

Finally, I would especially like to thank Brad Elmer for his contribution to the initial device design, Bill Stearns for his expertise and contribution to the device design modifications, Barry Haesly for providing ball slide samples, Sarita Bhetawal, Priscila Martinez, and Viridiana Ramirez for their assistance with surgical preparation and behavior testing, and Reuel Cornelia and Richard Bamlaygas at Texas Scottish Rite Hospital for Children for technical assistance in histological preparations.

I will give thanks to You, O Lord my God, with all my heart and will glorify Your name forever.

Psalm 86:12

April 8, 2013

ABSTRACT

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Scoliosis corrective surgery requires the application of multidirectional stress forces to the spinal cord, including those of distraction, in addition to the application of fixation rods for correction of the curved spine deformity. If excessive, spine distraction may result in the development of new neurologic deficits, some as severe as permanent paralysis. Intraoperative monitoring is used to alert the surgeon to possible complications; however, the complex nature of spinal cord injury (SCI) involves a primary mechanical insult to the tissue and/or vasculature that may go undetected by monitoring. This is followed by the activation of widespread secondary injury mechanisms. The specific injury mechanisms involved in distraction SCI remain largely unknown, increasing the difficulty of tailoring the selection of possible preventative therapeutics. The majority of animal models used to study the pathobiology of SCI are rat models of transection or contusion, though there have been a few previous models of distraction that have sought to characterize the injury. Deficiencies in the models combined with a lack of functional assessment, however, have limited their ability to effectively elucidate the injury mechanisms. To address such limitations, we designed a novel device that relies on intervertebral grip fixation and linear actuators to induce controllable bidirectional distraction injuries to the spine. The device was tested in three (i.e., 3, 5, and 7-mm) distention paradigms

of the rat T9-T11 vertebrae, and the resulting injuries were evaluated through electrophysiological, behavioral, and histological analysis. As expected, animals with 3-mm bidirectional spine distractions showed no neurological deficit. In contrast, those with 5 and 7-mm distractions showed partial and complete paralysis, respectively. The relationship between the severity of the spine distraction and injury to the spinal cord tissue was determined using glial fibrillary acidic protein immunocytochemistry for visualization of reactive astrocytes and labeling of ED1-positive activated macrophages/microglia. These results demonstrate that this device can produce bidirectional spine distraction injuries with high precision and control. To increase the clinical relevance of the model, we further modified the distraction paradigm in which the animal is held in a distracted position for a prolonged period. The observed injury was mild, as evidenced by a lack of reduction in transcranial motor-evoked potential amplitude, intact cords, and a mild behavioral deficit. For the purpose of elucidating the specific injury mechanisms, we then determined the extent to which distraction induces a hypoxic insult through direct measurement of the partial pressure of oxygen (pO_2). We recorded a transient sharp decline in pO_2 levels in the distal cord parenchyma immediately following the application of distractive force. This sharp decline was followed by a mild hypoxic insult for the duration of the time held in the distracted position. We also observed an acute increase in protein oxidation 30 minutes post-injury. Taken together, these results have led to a greater understanding of the injury mechanisms involved in distraction SCI that will better enable the tailoring of neuroprotective strategies aimed at preventing the onset of neurological deficits during spine deformity surgery.

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CHAPTER 1

INTRODUCTION

Spinal cord injury (SCI) is an unfortunate reality for many people with an annual incidence of 15 to 40 cases per million worldwide (Sekhon and Fehlings 2001). The nature of SCI is complex, involving the activation of varying physiological responses that lead to widespread cellular and molecular changes and result in devastating loss of neurological function below the level of insult. The widespread nature of the subsequent cellular and molecular changes increases the difficulty of tailoring the selection of possible therapeutic interventions; therefore, a comprehensive study of the specific injury mechanisms associated with SCI is a crucial first step toward elucidating rational therapeutic interventions.

1.1 Clinical Relevance

1.1.1 Spine Deformity

Spine deformity is generally classified by the type and severity of the deformity (Sucato 2010). Although kyphosis is a type of spine deformity, the focus of this dissertation is scoliosis correction. Scoliosis is a complex three-dimensional spine deformity with lordosis, lateral deviation, and axial rotation of the vertebral column caused by genetic or idiopathic disruptions that result in progressive spine curvature (Weinstein et al. 2008, Vitale et al. 2010). Patients are typically diagnosed with adolescent idiopathic scoliosis (AIS) when a Cobb angle of 10º or greater is evident on a radiograph and other known causes of spine curvature are rejected (Weinstein et al. 2008). One in a 1,000 children develops AIS which, if severe, compromises respiration and demands surgical correction (Carter and Haynes 1987, Sucato 2010).

1.1.2 Non-surgical Treatment for Spine Deformity

When an AIS curve exceeds 25º, the first method of treatment is bracing to mitigate further curve progression while skeletal maturity improves (Bradford and Tribus 1997, Parent et

al. 2005). Unfortunately, the effectiveness of bracing is subject to patient compliance, which is difficult to control. Clinicians gage the success or failure of bracing by whether a curve progression of more than 5° occurs during bracing, as well as by how many curves require surgical intervention despite treatment with bracing (Weinstein et al. 2008). The reported rate of surgical intervention despite bracing ranges from 7% to 43% depending on patient compliance and the type of brace used (Little et al. 2000, Trivedi and Thomson 2001, Weinstein et al. 2008).

1.1.3 Surgical Correction of Spine Deformity

Surgical correction of AIS typically involves the placement of rods fixed to screws that are secured into vertebral pedicles and the application of corrective forces, including distraction and rotation, to straighten the vertebral column, as shown in Figure 1.1 (Buchowski et al. 2006, Buchowski et al. 2007, Sucato 2010, Master et al. 2011). Risks associated with the surgical correction of scoliosis include pulmonary issues, intraoperative blood loss, direct contusion of the cord, and neurological deficits (Winter 1997, Weinstein et al. 2008, Master et al. 2011). The types of iatrogenic insults believed to contribute to SCI during scoliosis corrective surgery include: (a) cord compression due to vertebral translation, (b) cord kinking and dura buckling, (c) hypoxia secondary to segmental blood vessel ligation and decreased blood pressure, and/or (d) direct spinal cord stretching (Mcafee and Bohlman 1985, Gonzalez et al. 2009, Master et al. 2011).
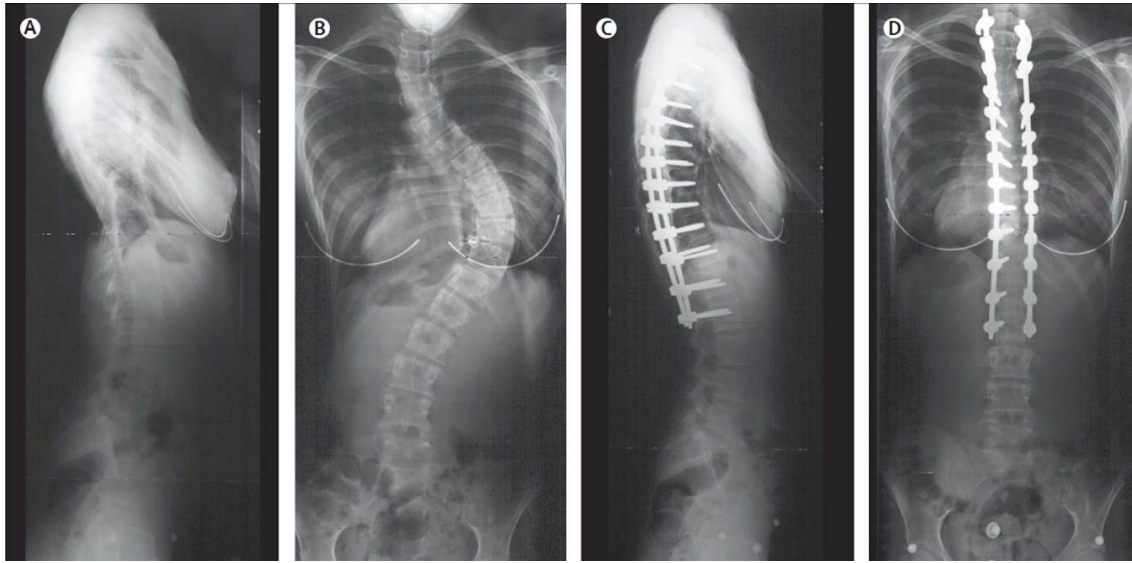
Figure 1.1 AIS case before and after surgical correction (Weinstein et al. 2008).

In cases of severe scoliosis, halo traction is often employed to aid in the alignment of the spine by lessening the severity of the curve prior to surgery, as shown in Figure 1.2A (O'brien et al. 1971, O'brien et al. 1973, Sink et al. 2001, Mehlman et al. 2004, Buchowski et al. 2006). More recently, temporary internal distraction, as shown in Figure 1.2B, has been suggested as an alternative when halo traction is not advised, such as in the case of cervical instability to prevent overdistraction and in cases of lumbar spine deformities when halo traction is not as effective; it is generally applied one to four weeks prior to corrective surgery depending on the severity of the curve (Buchowski et al. 2006, Buchowski et al. 2007).
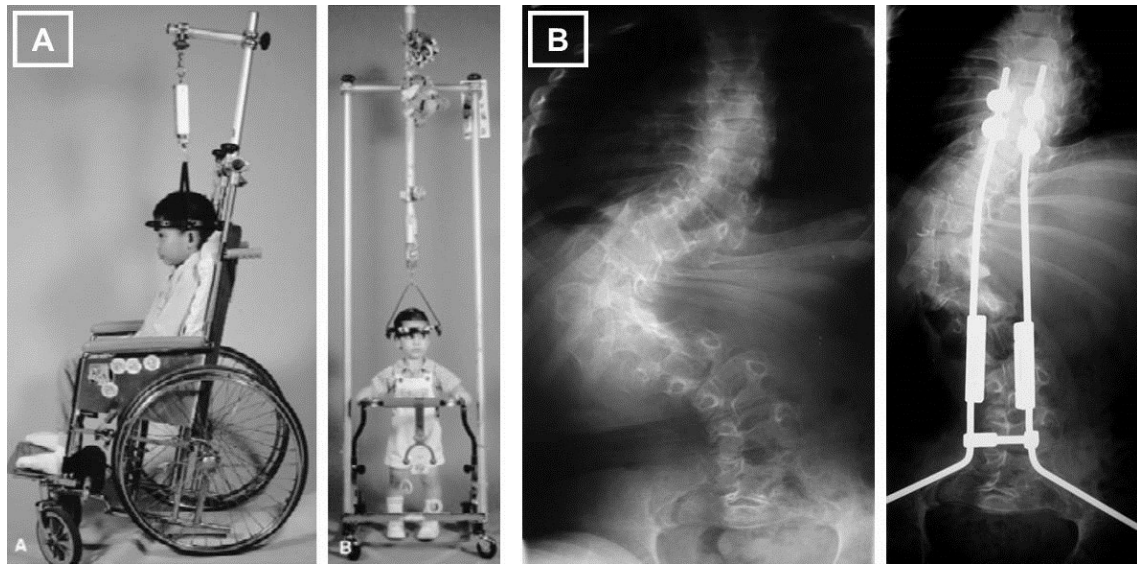
Figure 1.2 Methods commonly utilized to aid in the alignment of the spine prior to corrective surgery. (A) Halo-gravity traction (Sink et al. 2001). (B) Temporary internal distraction (Buchowski et al. 2006).

1.1.3.1 Intraoperative Monitoring

Intraoperative neurophysiological monitoring (IONM) is aimed at early detection of possible complications to allow for surgical intervention to mitigate unintended damage to the spinal cord (Gonzalez et al. 2009, Malhotra and Shaffrey 2010). Multi-modal monitoring of both somatosensory-evoked potentials (SSEPs) and transcranial motor-evoked potentials (TcMEPs) is often employed (Malhotra and Shaffrey 2010, Sucato 2010, Vitale et al. 2010). SSEPs are obtained by stimulating peripheral nerves and recording from the somatosensory cortex; conversely, TcMEPs are obtained by stimulating the motor cortex and recording from proximal and distal muscles, as shown in Figure 1.3.

Somatosensory-Evoked Potentials (SSEPs)

record from somatosensory cortex

stimulate peripheral nerve

— brain

— spinal cord

stimulate peripheral nerve

Transcranial Motor-Evoked Potentials (TcMEPs)

stimulate motor cortex

record from proximal muscle

— brain

— spinal cord

record from distal muscle
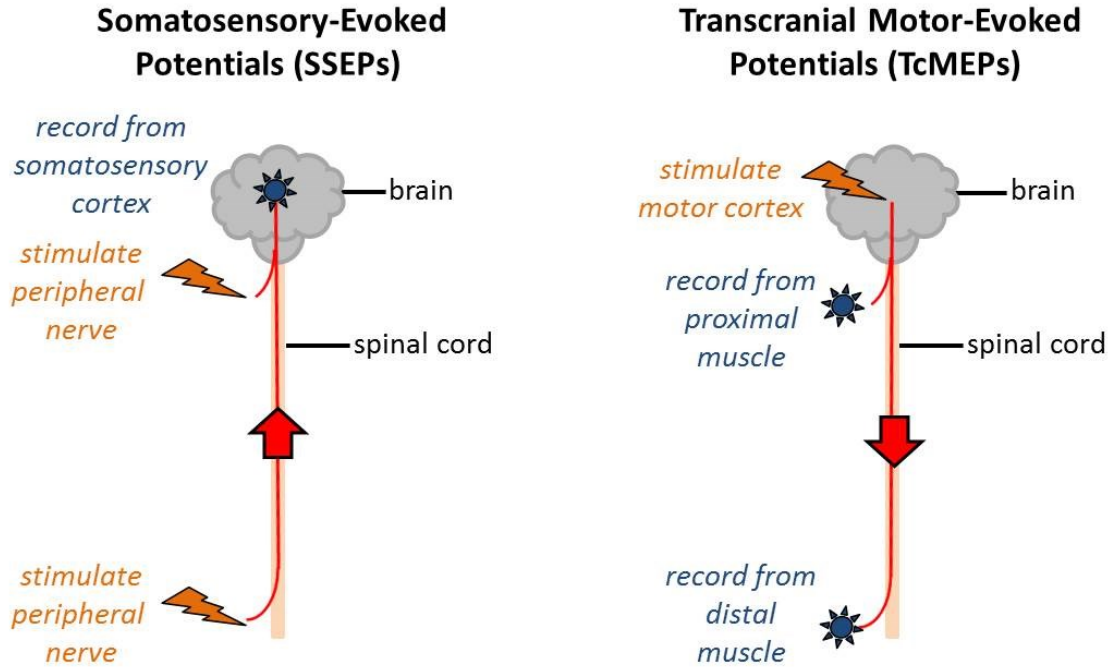
Figure 1.3 Schematic of somatosensory-evoked potentials (SSEPs) and transcranial motor-evoked potentials (TcMEPs).

Electrophysiological changes indicative of possible injury include a reduction in SSEP amplitude of >50%, an SSEP latency increase of >10%, or a loss or decrease in TcMEP amplitude of >75% (Malhotra and Shaffrey 2010, Vitale et al. 2010). An example of TcMEP loss and recovery is shown in Figure 1.4.
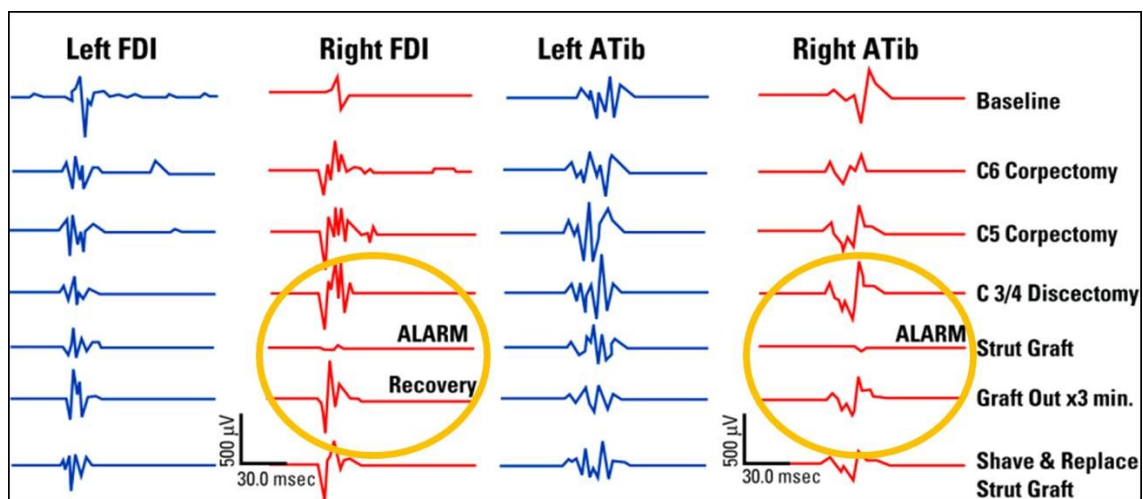
Figure 1.4 Example of TcMEP loss and recovery (Hilibrand et al. 2004). Motor potentials recorded from the first dorsal interosseous (FDI) and anterior tibialis (ATib) muscles of the hand and leg, respectively, are lost on the right side (yellow circles) following placement of a strut graft but are recovered following graft removal during cervical spine surgery.

1.1.3.2 Neurological Deficits

The incidence of neurological deficits is <1% following surgical correction of AIS and has been reported to be 5.7% to 6.3% in cases of severe spinal deformity (Macewen et al. 1975, Bradford and Tribus 1997, Coe et al. 2006, Lenke et al. 2009, Sucato 2010). Although rare, the most feared complication of scoliosis corrective surgery is paralysis (Winter 1997). As more severe curves are surgically corrected with more aggressive approaches, the incidence of paralysis may increase. For example, vertebral column resection (VCR), one of the most aggressive surgical methods used to mobilize the spine and chest to aid in the correction of severe spine deformity entails removal of the neural arches, vertebral body, and discs, as shown in Figure 1.5 (Sucato 2010, Enercan et al. 2013).

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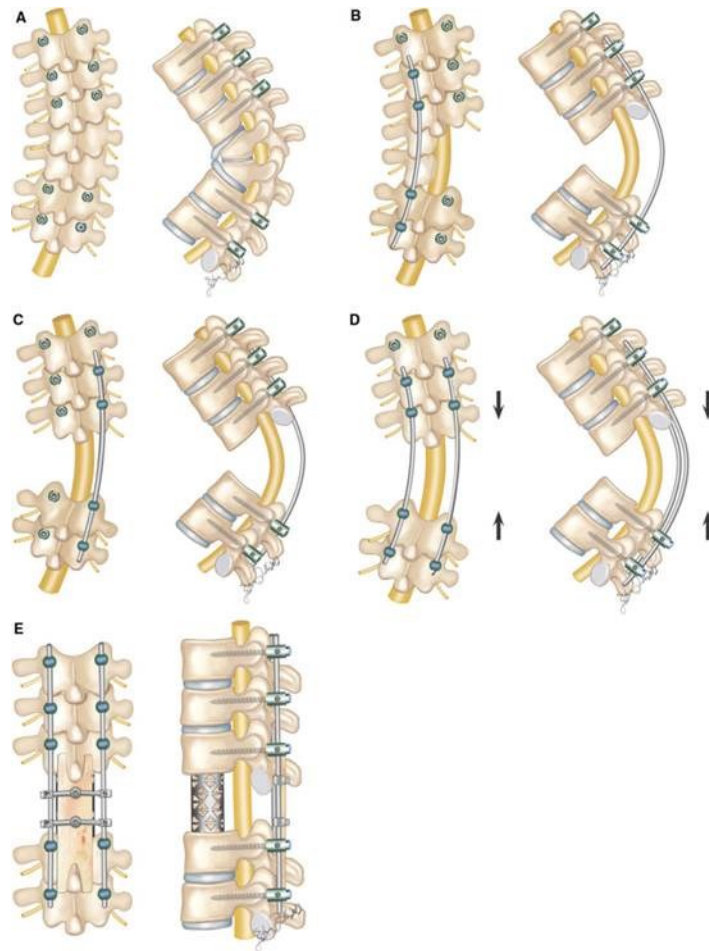
Figure 1.5 Illustration of posterior vertebral column resection (Enercan et al. 2013).

One study recently identified a 59% complication rate associated with VCR for the correction of severe spine deformity in which 27% of patients had either an IONM change or a failed wake-up test due to spinal cord or nerve root deficit while no cases resulted in permanent paralysis (Lenke et al. 2013). Thus, the incidence of complications that lead to neurological deficits following spine deformity surgery necessitates the need for an animal model capable of reproducibly mimicking the neurological deficits that can result from the application of distractive forces during spine deformity surgery for the purpose of providing valuable contributions to the understanding of the specific injury mechanisms involved in the onset of distraction-induced SCI

and determining the efficacy of treatment strategies aimed at reducing and/or preventing the incidence of new neurological deficits following scoliosis corrective surgery.

1.2 The Biphasic Nature of SCI

Damage to the spinal cord following SCI can be classified into two phases (primary and secondary injury) based on the pathophysiological and histological changes evident in the cord (Norenberg et al. 2004). The fact that pathophysiological changes in the spinal cord are often not observed within the first few hours following the initial injury suggests that the majority of the abnormalities evident at later time points are due to secondary injury mechanisms (Lipton and Rosenberg 1994, Norenberg et al. 2004).

1.2.1 Primary Injury

The primary injury refers to the initial physical insult to the spinal cord involving a mechanical disruption of the tissue (i.e., compromise of the structural integrity of the cord) and/or surrounding vasculature. It is difficult to isolate mechanical damage to the tissue and mechanical damage to the vasculature because they often occur concurrently (Dolan et al. 1980, Naito et al. 1992).

1.2.1.1 Mechanical Insults

The main types of mechanical insults to the cord are compression/contusion, distraction, and laceration/transection (Bunge et al. 1993, Oyinbo 2011). In terms of human SCI, while complete transection of the spinal cord is a rare occurrence, the most common mechanical insults are either cord compression or laceration resulting when bone fragments loosened by dislocation impinge the cord (Tator 1983, Norenberg et al. 2004, Borgens and Liu-Snyder 2012).

1.2.1.2 Effects on Vasculature

The primary insult can mechanically disrupt the vasculature of the cord, and this vascular compromise can occur in the presence or absence of tissue damage. (Dohrmann et al. 1971, Ducker et al. 1971, Griffiths and Mcculloch 1983, Hall et al. 1989). While the effects of

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vascular compromise following SCI will be discussed in more detail in subsequent sections, it is important to first consider vascularization of the spinal cord under normal conditions in order to understand the effects of vascular disruption following the initial injury. Arterial blood supply in the uninjured spinal cord can be classified into a central system, in which blood is supplied primarily by the anterior spinal artery, and a peripheral system, in which blood is supplied primarily by the posterior spinal artery and the pial arterial plexus, as shown in Figure 1.6 (Tveten 1976, Martirosyan et al. 2011). The percentage of blood supplied from the central versus the peripheral system, as well as overlap between the two systems, varies throughout the different regions in the cord (Hassler 1966, Martirosyan et al. 2011). Blood to the thoracic region, for example, is supplied predominantly by the peripheral system and less by the central system; moreover, the overlap between the two systems is poor compared to the collateralization in the cervical and lumbar regions, predisposing the thoracic region of the cord to ischemia following vascular compromise (Hassler 1966, Martirosyan et al. 2011).
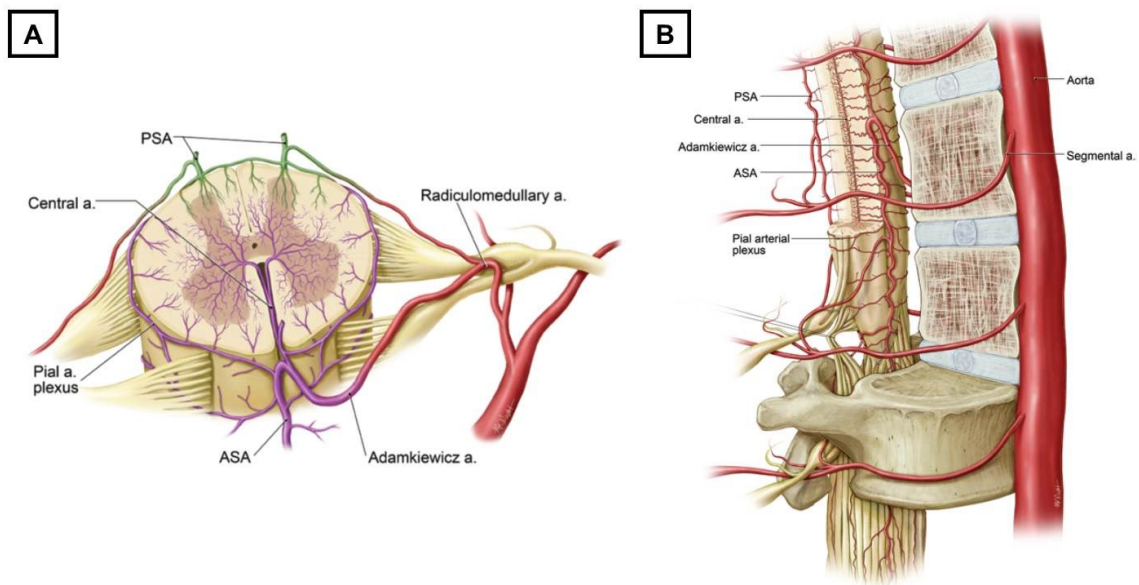
Figure 1.6 Illustration of blood supply to the lumbar spinal cord from a transverse view (A) and an anterolateral view (B) (Martirosyan et al. 2011).

It is well established that the mechanism of autoregulation in the spinal cord maintains constant blood flow through the cord through vasoconstriction and dilation during periodic changes in systemic blood pressure or CO_2 concentrations (Smith et al. 1969, Kindt 1971, Kobrine et al. 1975, Martirosyan et al. 2011). In 1975, Kobrine et al. sought to characterize the effects of arterial pCO_2 ($pACO_2$) and mean arterial pressure (MAP) changes on spinal cord blood flow (SCBF) in Rhesus monkeys using the hydrogen clearance technique. It was discovered that varying the $pACO_2$ away from the physiological level of 30-35 mm Hg within the range from 10 mm Hg to 50 mm Hg did not induce changes in SCBF; however, $pACO_2$ values between 50 to 90 mm Hg caused an increase in SCBF (Kobrine et al. 1975). It was also learned that changes in MAP between 50 and 135 mm Hg did not induce changes in SCBF, yet MAPs below 50 mm Hg or above 135 mm Hg disrupted autoregulation and led to a decrease or increase in SCBF, respectively (Kobrine et al. 1975). From these findings, Kobrine et al. (1975) then expressed SCBF by the following equation to define the mechanism of autoregulation in the spinal cord in terms of MAP and vascular resistance (VR): SCBF = MAP/VR. From this relationship, one can understand how changes in MAP and/or VR due to occlusion and/or rupture of the vasculature can result in loss of autoregulation and, thus, changes in SCBF, ischemic injury, hemorrhaging, and edema.

1.2.2 Secondary Injury

The notion of secondary injury was first postulated in 1911 when it was observed that draining the blood and serum from the injured region of a dog's spinal cord following a contusion injury improved the animal's recovery; it was hypothesized that a "biochemical irritation" in the fluid exacerbated the injury (Allen 1911, 1914). Since then, researchers have focused on elucidating the specific secondary injury mechanisms and understanding the sequence of those events following acute traumatic SCI. From these studies, it has been well established that the primary injury, in addition to inducing immediate cell death and/or vascular compromise upon impact, leads to the activation of secondary signaling cascades that result in

widespread cellular and molecular changes that culminate in necrotic and/or apoptotic cell death (Tator and Fehlings 1991).

The onset of secondary injury mechanisms can be divided into three phases: acute, sub-acute, and chronic, as shown in Figure 1.7 (Oyinbo 2011). While the scope of secondary injury mechanisms known to occur following SCI is vast, the focus of this dissertation is on the prominent acute and sub-acute injury mechanisms highlighted in Figure 1.7 and described in detail below.

Primary Injury
Compression/Contusion
Laceration/Transection
Distraction

Minutes ⎯⎯ Weeks ⎯⎯ Months ⎯⎯ Years ⎯⎯→

Acute
Necrotic cell death from direct insult
Ischemia
Edema
Accumulation of neurotransmitters

Sub-acute
Cell death from direct insult
Ischemia/hypoxia
Edema
Energy failure and decreased ATP
Glutamate excitotoxicity
Free-radical production
Protein/lipid peroxidation
Nitrous oxide excess
Inflammatory mediated cell death
Vetebral compression/column instability
Demyelination of surviving axons
Apoptosis
Astroglial scar launch

Chronic Secondary Injury
Continued demyelination
Continued apoptosis
Glial scar formation
Regenerative processes

Figure 1.7 Timeline of onset of various secondary injury mechanisms, adapted from (Oyinbo 2011). Specifically, ischemic injury leads to impaired energy metabolism and decreased energy production, which in turn leads to glutamate excitotoxicity and oxidative stress mechanisms.

1.2.2.1 Vascular Disruption

As mentioned earlier, the mechanical trauma to the cord can disrupt the microvasculature, leading to hemorrhage, ischemia, and edema (Norenberg et al. 2004, Borgens and Liu-Snyder 2012). The rupturing of blood vessels leads to hemorrhaging at the injury site following SCI. Studies have observed increased hemorrhaging in the gray matter compared to that in the white matter, which has been hypothesized to result because of the

dense capillary network in the gray matter (Vlajic 1978, Sasaki 1982, Tator and Koyanagi 1997). Thus, the extent of vascular damage in a given region depends not only on the severity of the injury but also on the normal pre-injury vascularization of that region. It has also been shown through several studies that the initial mechanical damage to the spinal cord vasculature results in an immediate reduction in SCBF, leading to ischemia and, thus, decreased oxygen in the surrounding tissue (Bingham et al. 1975, Kobrine et al. 1975, Griffiths 1976, Ducker et al. 1978, Senter and Venes 1978, Hayashi et al. 1980, Fehlings et al. 1989, Tator and Fehlings 1991). In particular, the gray matter has been found to be more susceptible to this ischemic damage compared to the white matter (Kobrine et al. 1975, Ducker et al. 1978, Rivlin and Tator 1978, Holtz et al. 1989). Currently, it is unknown whether this onset of ischemia is a result of systemic hypotension or disruption to the mechanism of autoregulation (Guha et al. 1989, Schwab and Bartholdi 1996, Rowland et al. 2008). In addition to hemorrhaging and ischemia, another deleterious consequence of vascular compromise is vasogenic edema, which can be induced by disruption of the blood-brain barrier (BBB), leading to fluid leakage into the extracellular space (Griffiths and Miller 1974, Norenberg et al. 2004). The onset of vasogenic edema has been observed following both compression and contusion injuries (Beggs and Waggener 1976, Noble and Wrathall 1989). Cytotoxic edema, or intracellular swelling, has also been observed in astrocytes following injury (Kimelberg 1992).

1.2.2.2 Disruption of Ionic Homeostasis and Glutamate Excitotoxicity

It has been shown that impaired energy (ATP) metabolism resulting from ischemia can lead to the failure of ATP-dependent ion transport pumps; the resulting depolarization leads to increased intracellular levels of calcium, which promote the synaptic release of glutamate such that extracellular concentrations of glutamate reach toxic levels within minutes following SCI, leading to increased firing of neurons (Liu et al. 1991, Lipton and Rosenberg 1994, Iwata et al. 2010). The abnormally high levels of glutamate then lead to the excessive activation of sodium and calcium-permeable glutamate receptors, mainly the NMDA-receptor; this results in an

accumulation of intracellular sodium followed by calcium that further promotes the release of glutamate (Taylor et al. 1992, Lipton and Rosenberg 1994). It has been hypothesized that this sequence of events induces intracellular swelling followed by delayed degeneration in neurons, eventually culminating in apoptotic neuronal cell death (Olney et al. 1986, Choi 1988, Hazell 2007).

1.2.2.3 Free Radical Production and Oxidative Stress

Under normal conditions, aerobic cellular respiration produces free radicals which are normally controlled by endogenous antioxidants, such as superoxide dismutase (SOD) (Halliwell and Cross 1994, Phillis 1994). Following ischemic injury, the reduction in the availability of oxygen takes away the normal terminal electron receiver in the electron transport chain, leading to an increase in reactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, and hydroxyl radical. Mitochondrial respiration is further impaired by the rise in intracellular calcium levels and the resulting glutamate excitotoxicity described above, leading to further generation of ROS (Happel et al. 1981, Moriya et al. 1994). This creates a cellular imbalance between free radicals and the ability of endogenous antioxidants to maintain normal levels of those free radicals, resulting in oxidative stress (Frei 1994, Turrens 2003). ROS are generated early following SCI and cause further cellular damage by inducing lipid peroxidation and oxidative damage to nucleic acids and proteins (Luo et al. 2002, Xu et al. 2005).

1.2.2.4 Inflammation

The role of the inflammatory response following SCI remains a controversial topic. On one hand, inflammation is beneficial in that it facilitates the removal of cellular debris at the site of injury; however, it can actually result in exacerbation of the initial injury, as well as damage to uninjured tissue nearby, if it is not controlled (Donnelly and Popovich 2008). It is known that inflammation is an immediate response following SCI that can last from weeks to months (Fehlings and Nguyen 2010). Microglia, the innate immune cells found in the central nervous

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system (CNS), are immediately activated and begin to secrete pro-inflammatory cytokines, such as tumor necrosis factor (TNFα), interleukin (IL)-1β, and IL-6, which recruit more inflammatory cells to the injury site, thereby propagating the inflammatory response (Fehlings and Nguyen 2010). Neutrophils are recruited to the injury site within the first day post-injury and have been shown to secrete pro-inflammatory cytokines, as well as proteases and reactive oxygen species (ROS), which can lead to the damage of nearby endothelial cells (Harlan 1987, Carlos and Harlan 1994). Interestingly, it has been shown that decreasing the interaction between neutrophils and endothelial cells can lessen the severity of a compression injury (Harlan 1987). Monocytes are also recruited to the injury site at a later time point (3-7 days post-injury) after which they differentiate into macrophages and also begin secreting pro-inflammatory cytokines and ROS (Lindholm et al. 1992). These pro-inflammatory cytokines and ROS further exacerbate the inflammatory response and oxidative stress mechanisms.

1.2.2.5 Overlap of Secondary Injury Mechanisms

The difficulty in elucidating the specific injury mechanisms associated with a given injury paradigm lies in the great deal of overlap between the various secondary injury mechanisms, as shown in Figure 1.8. However, without a proper understanding the specific injury mechanisms, it is difficult to tailor the selection of possible preventative therapeutic strategies for a given injury paradigm.
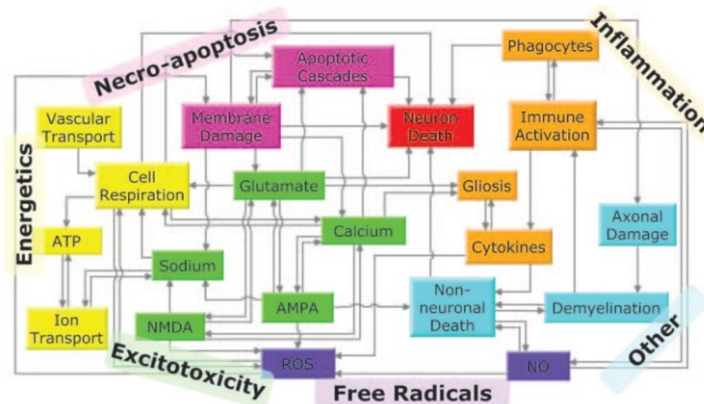
Figure 1.8 Diagram showing the overlap between the various secondary injury mechanisms following SCI (Mitchell and Lee 2008).

<div align="center">1.3 Animal Models of SCI</div>

Animal models are routinely established to study individual injury paradigms and determine the efficacy of various treatment strategies. Injuries to the human spinal cord, however, are usually the result of multiple injury paradigms, making them difficult to replicate reproducibly in animal models. Nevertheless, studying the individual injury paradigms lends insight into the specific injury mechanisms involved in each paradigm. The majority of animal models currently used to study the pathophysiology of SCI are models of transection and contusion, as demonstrated by a literature search on PubMed (Figure 1.9). Although cord compression is hypothesized to contribute to SCI during scoliosis corrective surgery (Mcafee and Bohlman 1985, Gonzalez et al. 2009, Master et al. 2011), distraction is an equally relevant injury paradigm in the clinic that remains under-studied.
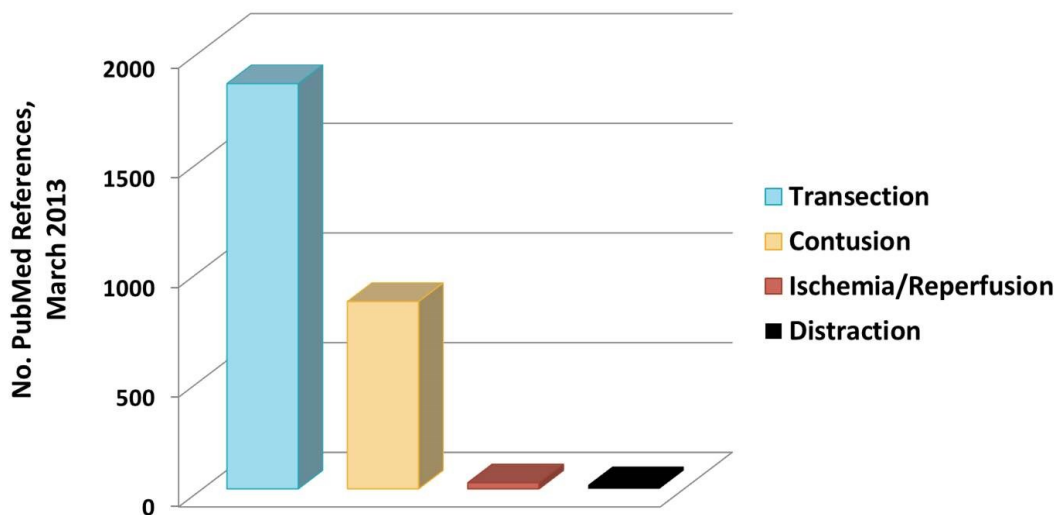
Figure 1.9 Comparison of the number of references on transection, contusion, ischemia/reperfusion, and distraction injury animal models on PubMed as of March 2013.

1.3.1 Models of Transection

Although complete transection rarely occurs in humans (Tator 1983, Norenberg et al. 2004, Borgens and Liu-Snyder 2012), models of complete and partial transection have been

used heavily to study axonal degeneration following SCI, as well as to evaluate the effectiveness of various regeneration strategies in the CNS. Transection results in mechanical damage to both the spinal cord tissue and vasculature (i.e., complete or partial severing of axons and blood vessels at the injury site) (Mcdonough and Martinez-Cerdeno 2012). Unfortunately, creating a partial transection injury, such as a dorsal or lateral hemisection, reproducibly has proved to be a rather difficult task (Onifer et al. 2007).

1.3.2 Models of Contusion

Contusion models simulate compression injuries in humans that result when dislocated vertebral bodies impinge the cord. The first model of contusion SCI was reported in 1911 when a weight was dropped from a known height onto the dorsal surface of the spinal cord of a dog (Allen 1911, 1914). This weight drop technique has since been applied to the rat, and subsequent studies have evaluated the effect of different weights and heights on injury outcomes (Wrathall et al. 1985, Panjabi and Wrathall 1988). The severity of the injury depends on the force applied to the spinal cord. Improved impactor devices have been established to afford more control over injury induction (Gruner 1992, Scheff et al. 2003).

1.3.3 Models of Ischemia/Reperfusion

Models of ischemia/reperfusion injury have been used to study vascular compromise in the spinal cord in the absence of tissue damage, such as following a descending thoraco-abdominal aortic aneurysm and subsequent surgical repair (Black and Cambria 2006). Studies using aortic cross-clamping to induce an ischemic injury in the spinal cord have shown that ischemia results in neurologic deficit, including paralysis, that is exacerbated by reperfusion (Kato et al. 1997, Oruckaptan et al. 2009, Liang et al. 2012).

1.3.4 Models of Distraction

Models of distraction have been used to mimic the application of distractive forces to the spine, such as during spine deformity surgery. Briefly, when the spinal column is stretched, it is hypothesized that the mechanical force is transmitted to the spinal cord through the spinal

nerves that extend from the spinal column and tether the cord. Despite the clinical incidence of flexion/distraction injuries, distraction SCI remains an under-studied injury paradigm in that there have been few previously published models. The first models to be published focused on determining the effect of distraction on SCBF for the purpose of evaluating the extent of vascular compromise. One model published in 1980 utilized a manually-operated apparatus to induce a unidirectional lumbar spine distraction injury in a cat, as shown in Figure 1.10 (Dolan et al. 1980). Spinal-evoked responses (SERs) were monitored to correlate IONM changes with changes in SCBF detected using the ^{14}C-antipyrine autoradiographic technique. It was shown that distraction, when associated with a noticeable change in somatosensory and/or motor-evoked potentials, leads to a reduction in blood flow without compromising the structural integrity of the cord (Dolan et al. 1980). While this study provided preliminary insight into the nature of the primary injury associated with distraction SCI, the model does not provide a reliable means to study the injury mechanisms further and to provide a platform on which to screen neuroprotective strategies.
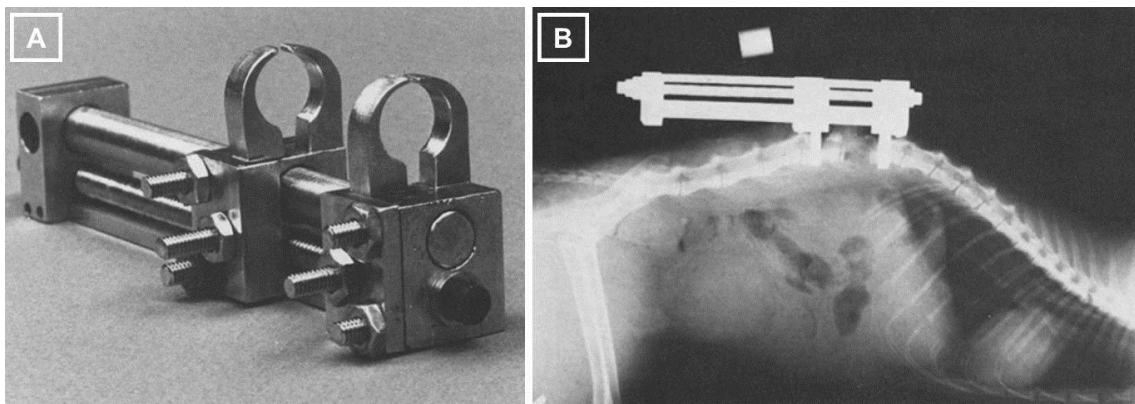
Figure 1.10 Early model of distraction SCI utilizing manually-operated apparatus (Dolan et al. 1980). (A) Manually-operated apparatus used to induce a unidirectional lumbar spine distraction injury in a cat. (B) Radiograph showing apparatus attached to L2 and L3 vertebrae post-injury.

Another group confirmed a reduction in SCBF dependent upon the nature of the IONM changes following distraction in a model utilizing Kostuik screws and bilateral Harrington rods to

induce spine distraction in a hog (Owen et al. 1990, Naito et al. 1992, Kai et al. 1993). Unfortunately, no functional assessment was performed aside from a wake-up test immediately before sacrifice at the end of the procedure.

More recently, two models were published that sought to induce a distraction injury more reproducibly; however, deficiencies in the models with subsequent high variability in the data, in combination with a lack of characterization, have limited their ability to effectively elucidate the injury mechanisms and be used reliably for future screening of neuroprotective strategies. The first model, published in 2004, utilized sublaminar modified Harrington hooks pulled by a stepping motor to induce a bidirectional thoracic spine distraction injury in a rat, as shown in Figure 1.11A (Dabney et al. 2004). This method requires a partial laminectomy for sublaminar hook insertion which could cause unintended direct traumatic damage to the spinal cord. The functional results for 5 and 7-mm distractions show highly variable degrees of injury (i.e., 3 out of 8 animals showed no behavioral impairment following a 7-mm distraction), as shown in Figure 1.11B (Dabney et al. 2004).
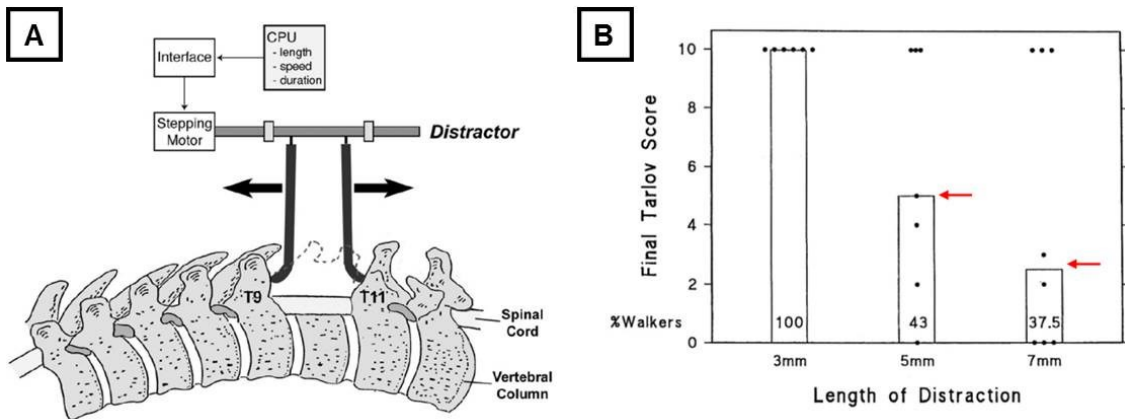
Figure 1.11 Model of bidirectional distraction SCI utilizing sublaminar modified Harrington hooks and a stepping motor (Dabney et al. 2004). (A) Diagram showing placement of sublaminar hooks pulled by a stepping motor. (B) High variability in the functional data (red arrows).

The second model, published in 2007, featured a device based on custom made clamps, with one clamp fixed and the other pulled by an electromagnetic linear actuator, that is

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able to induce a unidirectional cervical spine distraction injury in a rat, as shown in Figure 1.12 (Choo et al. 2007, Choo et al. 2008, Choo et al. 2009). Most distraction injuries, however, are presumed to be bidirectional, including those occurring during scoliosis correction. In addition, characterization of the injury only entailed limited histological analysis immediately post-injury; no long term functional assessment was performed.
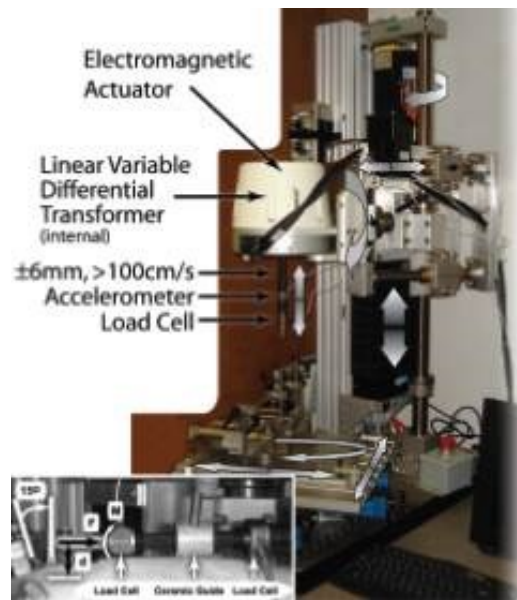
Figure 1.12 Model of unidirectional distraction SCI utilizing custom clamps and an electromagnetic linear actuator (Choo et al. 2007).

1.4 Hypotheses

The establishment and characterization of an animal model of distraction SCI that reproducibly mimics the neurological deficits that can result from the application of distractive forces during spine deformity surgery will provide valuable contributions to the understanding of the injury mechanisms involved in the onset of distraction-induced SCI. In addition, the model will serve as a platform on which to test neuroprotective strategies which could be implemented clinically to reduce the incidence of new neurologic deficits following scoliosis correction. As such, specific aims were designed to test the hypotheses that (1) a bidirectional distraction SCI will mimic the neurological deficits that can result from the application of distractive forces to the

spinal cord during scoliosis corrective surgery, as evidenced by characterization of the acute injury response, (2) a prolonged spinal distraction, in addition to the known mechanical forces, will introduce a hypoxic insult to the spinal cord that results in a recordable reduction in oxygenation levels in the distal cord parenchyma, and (3) the mechanical disruption and period of low oxygenation following distraction injury is sufficient to negatively affect mitochondrial function, leading to increased levels of reactive oxygen species (ROS) and an increase in protein oxidation.

1.5 Specific Aims

The following three specific aims were directed at (1) characterizing the acute effects of a graded distraction SCI, (2) evaluating the sub-acute effects of a clinically relevant distraction injury, specifically by determining the level of hemodynamic instability, and (3) determining the contribution of secondary injury mechanisms, specifically the level of oxidative stress, to the injury.

- *Specific Aim 1:* To characterize the acute injury response to a graded, bidirectional distraction SCI using the UTA Spine Distractor (Chapter 2). Here we confirmed a reduction in electrophysiological competency of the spinal cord, determined the extent of behavioral deficit, quantified the tissue loss, and evaluated the histological changes in response to a graded distraction injury.

- *Specific Aim 2:* To evaluate the sub-acute secondary effects of a clinically relevant distraction SCI (Chapter 3). Here we characterized the acute injury response to a prolonged bidirectional distraction spinal cord injury and monitored the partial pressure of oxygen (pO_2) before, during, and after injury in the spinal cord segments distal to the injury site to determine the extent to which distraction SCI induces low oxygen levels within the spinal cord.

- *Specific Aim 3:* To determine the contribution of secondary injury mechanisms to distraction SCI, specifically by examining the generation of reactive oxygen species

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(ROS) and protein oxidation (Chapter 3). Here we examined tissue homogenates from spinal cord sections obtained at various time points after distraction injury for levels of ROS formation and protein carbonyls (protein oxidation).

CHAPTER 2

CHARACTERIZATION OF A NOVEL BIDIRECTIONAL DISTRACTION
SPINAL CORD INJURY ANIMAL MODEL

2.1 Introduction

Acute traumatic SCI results in a devastating loss of neurological function below the level of insult. Based on the type of tissue damage, SCI can be categorized into contusion, distraction/flexion, dislocation, and partial or complete transection. Despite these varied mechanisms of insult, the majority of animal models available for studying the pathobiology of SCI have been performed primarily in rat models of spinal cord transection or contusion (Fehlings and Baptiste 2005, Samantaray et al. 2008, Anderson et al. 2009, Zhang et al. 2010).

Scoliosis is a complex three-dimensional spine deformity with lordosis, lateral deviation, and axial rotation of the vertebral column caused by genetic or idiopathic disruptions that result in progressive spine curvature (Weinstein et al. 2008, Vitale et al. 2010). One in a 1,000 children develops adolescent idiopathic scoliosis which, if severe, compromises respiration and demands surgical correction (Carter and Haynes 1987, Sucato 2010). This procedure involves the placement of rods fixed to screws that are secured into vertebral pedicles to straighten the vertebral column (Buchowski et al. 2006, Buchowski et al. 2007, Sucato 2010, Master et al. 2011). The incidence of neurological deficits is <1% following surgical correction of AIS and has been reported to be 5.7 to 6.3% in cases of severe spinal deformity (Macewen et al. 1975, Bradford and Tribus 1997, Coe et al. 2006, Lenke et al. 2009, Sucato 2010). Although rare, the most feared complication of scoliosis corrective spine surgery is paralysis (Winter 1997). The types of iatrogenic insults that contribute to SCI during scoliosis corrective surgery include: a) cord compression due to vertebral translation, b) cord kinking and dura buckling, c) hypoxia secondary to segmental blood vessel ligation and decreased blood pressure, and/or d) direct spinal cord stretching (Mcafee and Bohlman 1985, Gonzalez et al. 2009, Master et al. 2011).

The establishment and characterization of an animal model of distraction SCI that reproducibly mimics the neurological deficits that can result from the application of distractive forces during spine deformity surgery will provide valuable contributions to the understanding of the injury mechanisms involved in the onset of distraction-induced SCI, as well as a platform on which to test neuroprotective strategies which could be implemented clinically to reduce the incidence of new neurologic deficits following scoliosis corrective surgery.

Current animal models of distraction SCI include one based on sublaminar modified Harrington hooks pulled by a stepping motor, which creates highly variable degrees of injury (i.e., 3 out of 8 animals showed no behavioral impairment following a 7-mm distraction) (Dabney et al. 2004). This model also requires a partial laminectomy for sublaminar hook insertion, which could cause unintended direct traumatic damage to the spinal cord.

More recently, a device based on custom made clamps, with one clamp fixed and the other pulled by a linear actuator, was able to induce a unidirectional cervical spine distraction injury in a rat animal model (Choo et al. 2007, Choo et al. 2008, Choo et al. 2009). However, since most distraction injuries are presumed to be bidirectional, including those occurring during scoliosis correction, a need for an accurate and reliable model of spine distraction remains.

Here we report the development and testing of a novel spine distraction device which obviates the need for a laminectomy or sublaminar hooks, incorporates the use of bilateral computer-controlled linear actuators for distraction, and allows for electrophysiological monitoring, such as of TcMEPs and SSEPs. We hypothesized that a bidirectional distraction SCI will mimic the neurological deficits that can result from the application of distractive forces to the spinal cord during scoliosis corrective surgery. An animal model capable of mimicking these deficits would allow for the testing of neuroprotective strategies aimed at preventing unintended new neurological damage during corrective spine surgery. To test the hypothesis, we characterized the acute injury response to a graded, bidirectional distraction SCI using the UTA Spine Distractor. Specifically, we confirmed a reduction in electrophysiological competency

of the spinal cord, determined the extent of behavioral deficit, quantified the tissue loss, and evaluated the histological changes in response to a graded distraction injury.

<u>2.2 Materials and Methods</u>

Current available methods for modeling spine distraction injury rely on sublaminar hooks pulled by a stepping motor (Figure 2.1A) or are based on unidirectional spine distraction (Figure 2.1B). To better mimic the bi-directionality of distraction injuries that are present in most cases of SCI during scoliosis corrective surgery, we designed a device based on modified grips and two linear actuators that can reproducibly induce damage to the spinal cord through bidirectional spine distraction (Figure 2.1C).

Figure 2.1 Schematic comparison of different models of distraction spinal cord injury. (A) Bidirectional distraction using sublaminar hooks and stepping motor (Dabney et al. 2004). (B) Unidirectional distraction using clamps and linear actuators (Choo et al. 2007, Choo et al. 2008, Choo et al. 2009). (C) Bidirectional distraction using clamps and linear actuators (UTA Spine Distractor). (Seifert et al. 2011)

2.2.1 UTA Spine Distractor

The UTA Spine Distractor measures 24 X 10 X 6 inches, weighs 15 pounds and is made of five main components: structural support, guides, linear actuators, a load cell, and clamps. The structural components include a base plate (Figure 2.2A-V), a pair of side supports (Figure 2.2B- VII), sliding rods (Figure 2.2A-I), actuator connector plates (Figure 2.2A-III), and four undercarriage attachments (Figure 2.2B-VI). Each component was fabricated from aluminum and designed to hold a tolerance of ±0.01 inches. Side supports were connected vertically to the base plate with an opening in each side for easy access during animal placement. A sliding base, which can be freely moved between the two side supports and secured in place by bolts on both sides according to need, allows the operator to easily position the clamps for each distraction. Actuator connector plates were attached at opposites sides of the sliding base, and two electric linear actuators (NA11B30, Zaber Technologies Inc., Vancouver, Canada) were used to induce bidirectional linear distraction. Each actuator (Figure 2.2A-II) was connected to a stepper motor controller (T-MCA, Zaber Technologies Inc., Vancouver, Canada) and operated simultaneously using the manufacturer's software. The actuator's stroke length is up to 30 mm, can be retracted at 0.001-20 mm/s and accommodates a maximum continuous force of 80 N with approximate accuracy of 8 μm and repeatability of less than 0.4 μm.
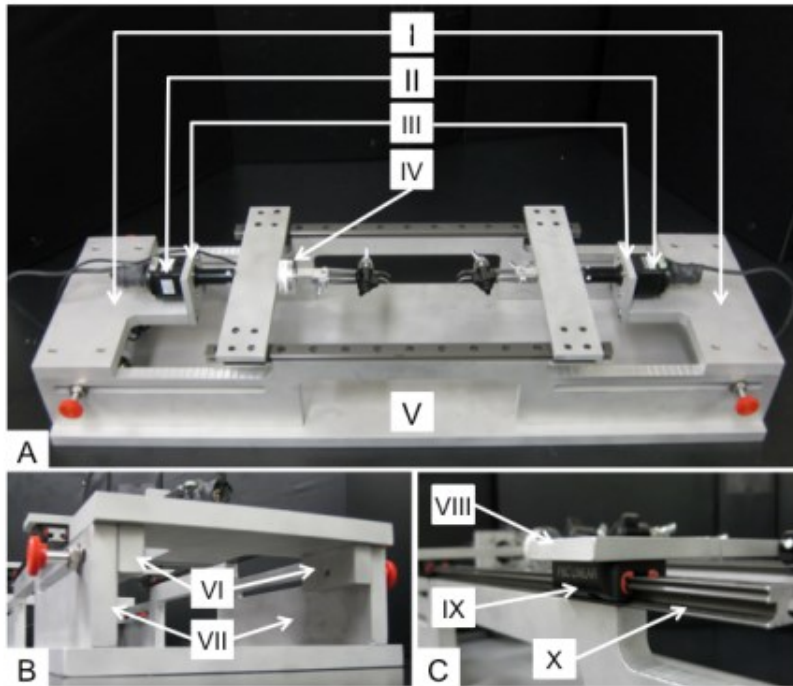
Figure 2.2 UTA Spine Distractor. (A) Top view of the UTA Spine Distractor showing the sliding bases (I), linear actuators (II), actuator connectors (III), load cell (IV), and base plate (V). (B) bottom view of the sliding base illustrating the undercarriage attachments (VI) and side supports (VII). (C) Detail of the side supports (VIII), guide blocks (IX), and guide rails (X). (Seifert et al. 2011)

Custom clamps were designed for placement at the intervertebral space, affixed to the vertebral body, and secured between the lateral spine processes (Figure 2.3). The clamps were made from surgical forceps (11003-12, Fine Science Tools Inc., Foster City, CA) that were separated and cut to 3-inch lengths. The clamps were fixed with a screw and wing nut, which allows them to be raised or lowered for easy positioning (Figure 2.3A-I and II). Each clamp was tightened around the spine using a knob and wing nut attached to flanges on the side of each clamp (Figure 2.3B-III). Approximately 6-8 serrations of the forcep clamps secure the vertebral body when the clamps are in place, thereby preventing the clamps from slipping during distraction (Figures 2.3C and 2.3D). The clamps used were optimized for the intervertebral space available in the rat spine.
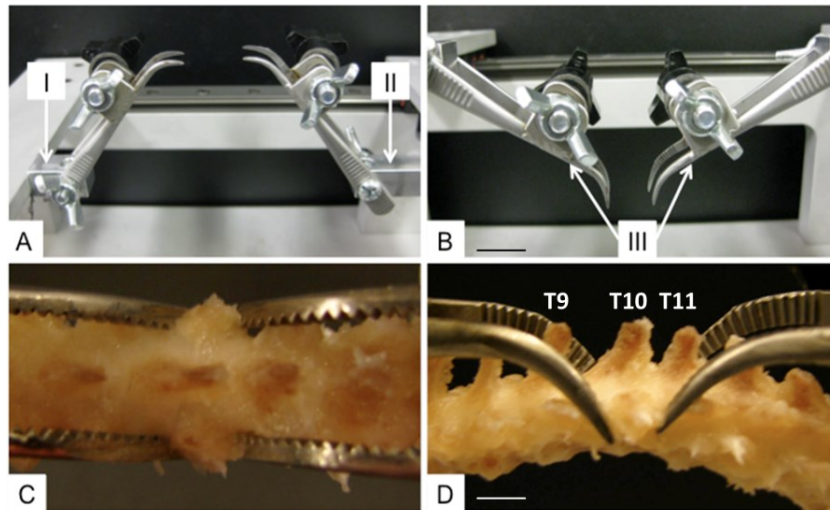
Figure 2.3 Vertebral body clamps. Customized serrated forceps were adapted to the UTA Spine Distractor for ease of placement over the vertebrae. Clamps can rotate up (A) and down (B) and are fixed with a threaded rod in the clamp block, one of which supports the load cell (I and II). Dorsal (C) and lateral (D) views of clamps placed over T9 and T11 vertebrae. Scale bar = 19 mm (B) and 4 mm (D). (Seifert et al. 2011)

To detect forces applied to the vertebral column during distraction, a capacitive load cell was used. The load cell (MFM-050-200-A*C03, Loadstar Sensors, Fremont, CA), which has a 50 lb. capacity and 0.5% accuracy at full scale, was firmly attached to one support piece (Figure 2.2A-IV) and connected to the computer via an interface (DQ-1000U, Loadstar Sensors, Fremont, CA). To eliminate the moment created by the weight of the load cell and clamps, two guide blocks (MRS12C-1C25, PBC Linear, Roscoe, IL) were attached to the bottom of each support piece and slid on to the guide rails (MRS12R-0500-R4, PBC Linear, Roscoe, IL). The guide blocks and rails have a very low coefficient of friction (0.1µ), thus ensuring a linear, bidirectional motion during distraction. The loadstar "LoadVUE" software was used to record distraction forces at a sample rate of 1 kHz. Peak recorded forces during distraction were 25.4 +/- 4.2 N.

The accuracy and reproducibility of the distraction device were confirmed by measuring the distracted distance using metal pins placed at the tips of the clamps. Actuators programmed

to move 7-mm averaged 7 ± 0.01 mm after 10 repetitions.

2.2.2 Experimental Design for Specific Aim 1

The first specific aim was directed at characterizing the acute injury effects of a graded, bidirectional distraction SCI using the UTA Spine Distractor. Specifically, we confirmed a reduction in electrophysiological competency of the spinal cord, determined the extent of behavioral deficit, quantified the tissue loss, and evaluated the histological changes in response to a graded distraction injury, as shown in Figure 2.4.
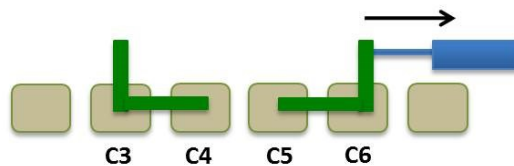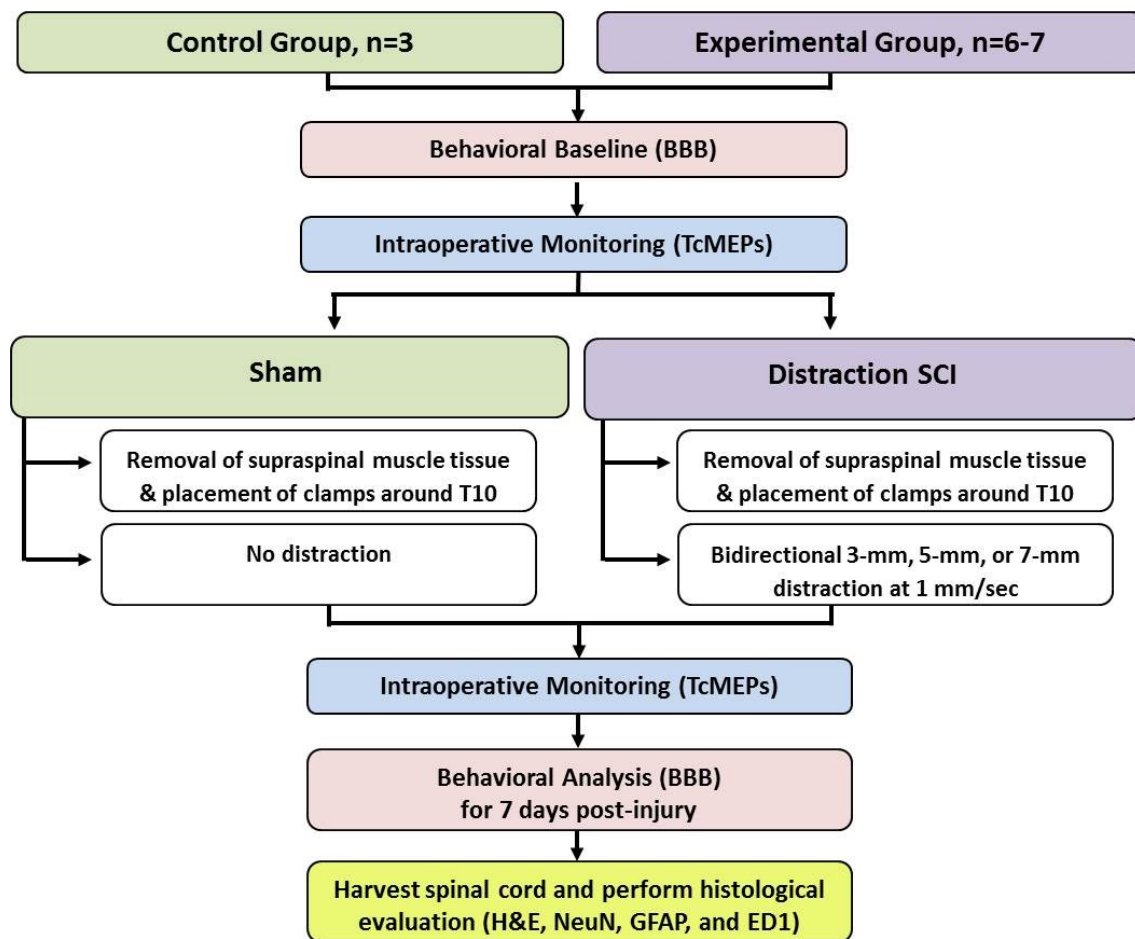

Figure 2.4 Experimental design for Specific Aim 1.

2.2.3 Distraction Spinal Cord Injury of Adult Rats

A total of twenty-three female Long-Evans rats (275-325 g) divided in three distraction groups: mild (3-mm), moderate (5-mm) and severe (7-mm) distractions were used for this study. The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). When an adequate depth of anesthesia was attained (loss of corneal reflex), the shaved dorsal surface was cleaned with povidone-iodine. The vertebral column was exposed by dissection of the paraspinous muscles and transection of the interspinous and facet capsular ligaments between T9/T10 and T10/T11. The vertebral clamps were placed in the T9 and T11 intervertebral spaces, and gentle tension was applied to ensure proper alignment of the vertebrae. The device was then tested at the specified distances: 3-mm (n=7), 5-mm (n=7), and 7-mm (n=6) at 1 mm/sec. After distraction, the clamps were removed, the muscle layer was approximated using chromic gut sutures, and the skin was stapled. As negative controls, some animals received a sham injury in which all procedures were consistent with distraction surgery, including clamp placement and the application of gentle tension, but no force was applied to induce distraction (n=3). All animals received antibiotic (cephazolin; 5 mg/kg, intramuscular) and pain control (buprenorphine; 0.05-0.1 mg/kg, subcutaneous) treatment post-surgery. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Texas at Arlington.

2.2.4 Electrophysiological Monitoring

Recording of TcMEPs were obtained after clamp placement and initial gentle tension (baseline), and immediately following spine distraction. Stimulating electrodes were placed over the motor cortex, and recording electrodes were placed in the right and left deltoid (above the lesion, negative controls) and gluteal (below the lesion, experimentals) muscles, as shown in Figure 2.5.
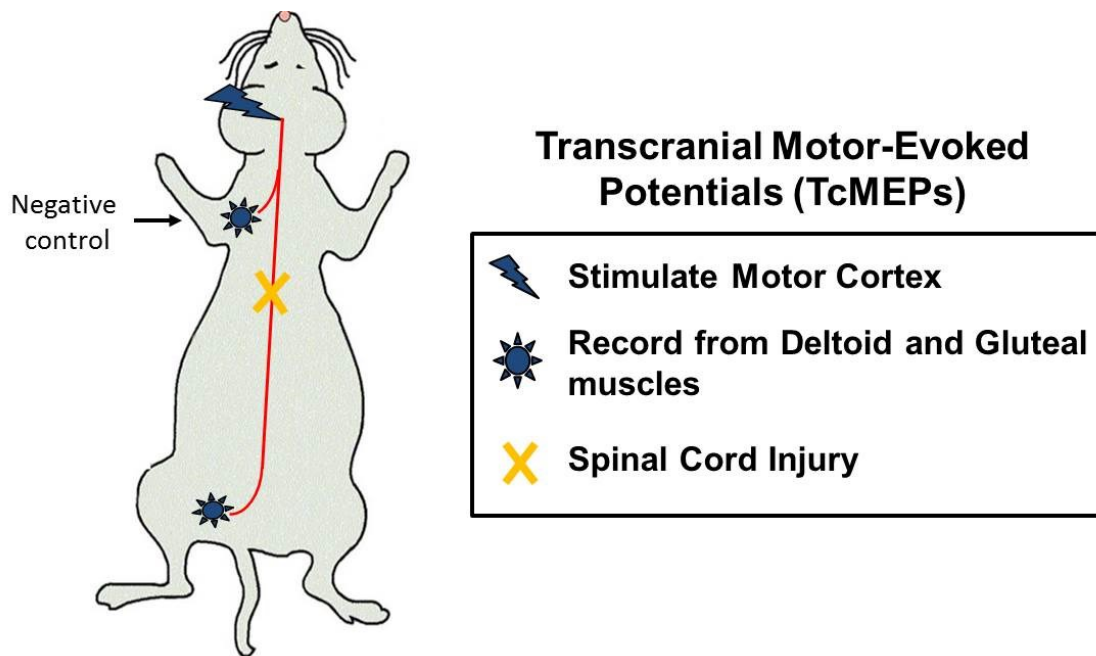
Figure 2.5 Schematic of TcMEP monitoring. Stimulating electrodes are placed over the motor cortex, and recording electrodes are located in the right and left deltoid (above the lesion, negative controls) and gluteal muscles (below the lesion, experimentals).

Reference electrodes were inserted in all four paws, and a ground electrode was placed in the ventral thigh. A Cadwell Cascade™ system was used to evoke motor activity by applying a train of three stimulation pulses (6-8 V amplitude, 50-μs duration, and 2-ms interstimulus interval) over the motor cortex. TcMEP signals were recorded at a rate of 21,500 samples per second. Low (≤100 Hz) and high (≥ 2 kHz) frequency components were filtered out, and the rest was amplified, processed and stored.

2.2.5 Behavioral Analysis

Behavioral analysis for motor function was performed using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al. 1995, Basso et al. 1996). This 22-point scale is used to assess gait, including front limb-hind limb coordination and plantar stepping. Absence of observable hindlimb movement is scored 0, and the initial points are awarded for isolated joint movements. The score increases when more joints show movement and/or the movements are more extensive. As locomotion increases, points are given for plantar

placement of the paw, weight support and coordination between forelimb and hindlimb. The final points are achieved by toe clearance, trunk stability and tail position. Each animal was acclimated to an open field tester for five minutes prior to a five-minute observation period during which two observers blinded to the groups assigned a BBB score based on the specific variables mentioned above, as shown in Figure 2.6. Behavioral baselines were established seven days prior to injury, and behavior was assessed daily for one week following injury.

A.

B.
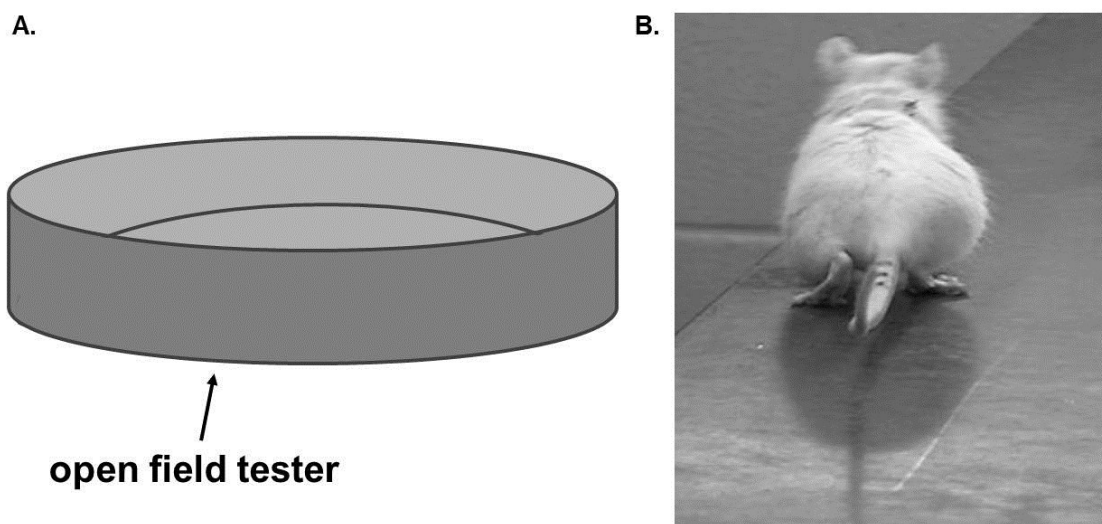
open field tester

Figure 2.6 Setup for BBB testing. (A) Each animal was acclimated to an open field tester for five minutes prior to the start of testing. (B) Each animal was observed for five minutes, after which a BBB score was assigned based on specific variables, including the animal's limb movement, trunk and abdomen position, paw placement, coordination, trunk stability, and tail placement (Loy et al. 2002).

2.2.6 Histological Analysis

Animals were perfused transcardially with 4% paraformaldehyde and the spinal cords harvested. Tissue was fixed overnight and processed for paraffin embedding. A 15-mm segment containing T9–T11 was isolated and divided into three 5-mm segments corresponding to proximal, injury epicenter, and distal sections. Transverse sections were cut and stained with hematoxylin and eosin (H&E). Percent tissue loss per section was determined by taking a ratio of the area of tissue loss over the area of the entire spinal cord section. Neuronal bodies were

visualized by staining for a neuronal marker (NeuN; 1:200 Mouse anti-NeuN; Millipore, Billerica, MA). The extent of tissue damage was ascertained by the visualization of reactive astrocytes and activated macrophages labeled with antibodies against glial fibrillary acidic protein (GFAP;1:500 Mouse anti-GFAP; Dako, Carpinteria, CA) and ectodermal dysplasia 1 (ED1;1:200 Mouse anti-CD68; AbD Serotec, Raleigh, NC), respectively. Deparaffinized tissue sections were blocked with endogenous peroxidase with 3% H_2O_2 and incubated in universal blocking solution (Biogenex; San Ramon, CA) prior to binding to peroxidase-conjugated antibodies (1:500; Jackson ImmunoResearch; West Grove, PA). Immunodetection of these markers was visualized by DAB (Dako, Carpinteria, CA).

2.2.7 Statistical Analysis

Decreases in TcMEP amplitude and the degree of tissue loss after SCI were quantified and evaluated via Kruskal-Wallis and pair wise comparisons. The BBB scores were analyzed using a two-way ANOVA (i.e., days post injury/distraction distance) followed by Bonferroni's post-hoc test. An α level ≤ 0.05 was considered significant.

2.3 Results

2.3.1 Graded Distraction Injury Induces Several Degrees of Functional Impairment

The TcMEP electrophysiological data recorded before and after distraction showed preserved function proximal to the lesion (deltoid muscle negative controls, data not shown) and several degrees of reduction in motor-evoked potential amplitudes recorded distal to the lesion (gluteal muscle) depending on the severity of the distraction injury. TcMEPs were not affected significantly by a 3-mm distraction but were significantly reduced (>75% drop in amplitude) in 40% of the animals distracted at 5-mm and in all subjected to a 7-mm distraction injury. Representative traces for each distraction distance are shown in Figure 2.7.
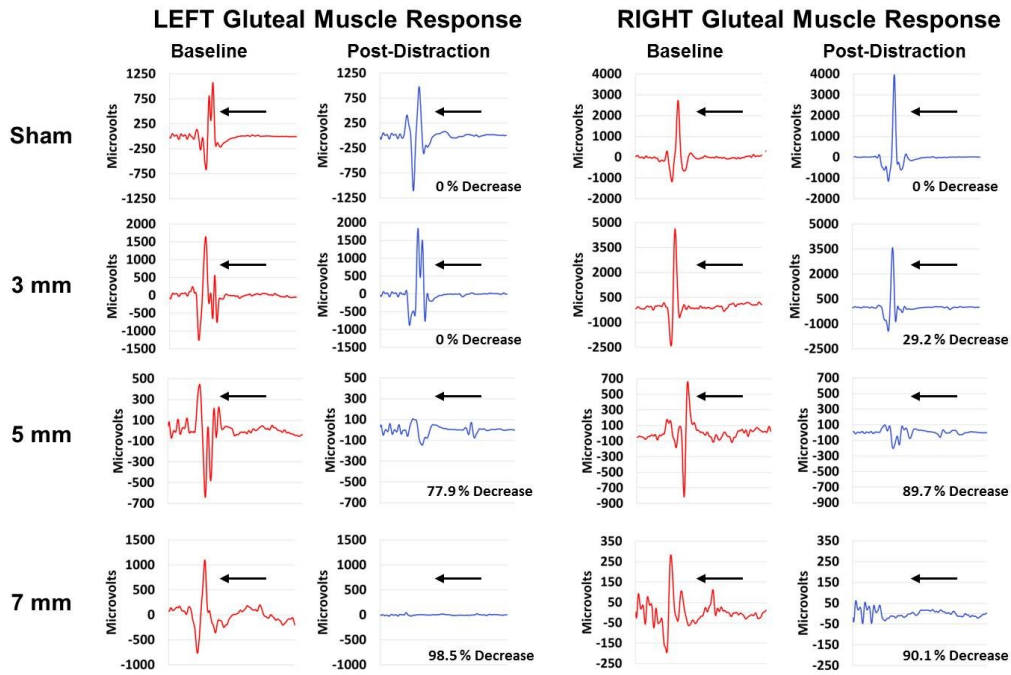
Figure 2.7 Representative TcMEP recordings below the lesion following a graded bidirectional distraction injury. The effect of mild (3-mm), moderate (5-mm) and severe (7-mm) injury causes a proportional reduction in evoked motor potentials (arrows).

Post-injury responses were compared to a baseline assessment for each animal. The differences were significant at both 5-mm and 7-mm compared to either sham injury or 3-mm distractions ($p \leq 0.05$). Specifically, the moderate distraction (5-mm) produced an average amplitude decrease of 58.7%, while the severely lesioned (7-mm) group showed an average drop of 85.8% (Fig. 2.8). Some animals in the sham and 3-mm groups exhibited an increase in amplitude; whereas, some exhibited an insignificant amplitude decrease. This was most likely related to the level of anesthesia, in addition to variability among animals.
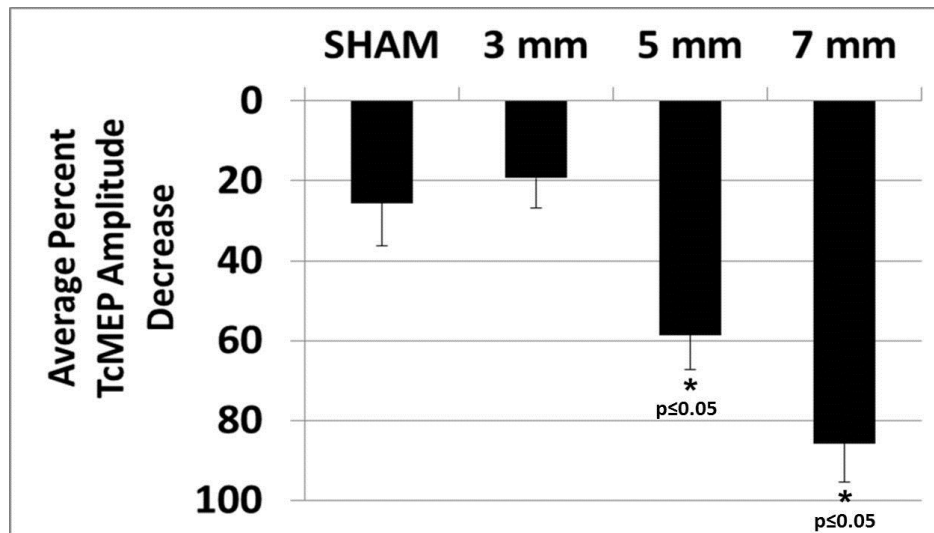
Figure 2.8 The TcMEP amplitude reduction is proportional to the distraction length. Quantification of average TcMEP amplitude decrease over graded injury revealed that motor-evoked potentials do not decrease following a 3-mm injury; however, they are reduced significantly following a 5-mm injury and are almost completely lost immediately following a 7-mm injury. Data represent the average and standard error of the mean. * $p \leq 0.05$ compared to sham/3-mm distraction. (Seifert et al. 2011)

Results from the behavioral analysis were also characteristic of a graded injury, as shown in Figure 2.9. Consistent with the intraoperative neural monitoring data, animals that underwent mild distraction (3-mm) showed no neurological deficits for one week following injury. Those with moderate distractions (5-mm) showed highly variable functional deficits (i.e., 0-18 BBB scores), while all severely distracted (7-mm) animals showed a dramatic reduction from a normal BBB locomotion score of 21 prior to distraction to a significantly lower score of 0 or 1 twenty four hours after injury (0.17; $p \leq .0001$). The functional deficit caused by the 7-mm distraction injury was highly reproducible, drastic, and permanent, in which none of the animals exhibited hindlimb movements. These results contrast that of previous reports in which a similar distraction paradigm using modified Harrington hooks produced a highly variable injury (approximately 50%) with no functional deficit observed in some animals. The distraction device reported here showed a variability of approximately 5%, that is, a 10-fold improved performance over previous methods (Dabney et al. 2004). The improved consistency in the induction of

complete SCI after severe distraction might be explained by the improved linearity of the applied forces and the bidirectional nature of the distraction induced by the UTA Spine Distractor compared to other methods (Dabney et al. 2004, Choo et al. 2007, Choo et al. 2008, Choo et al. 2009).
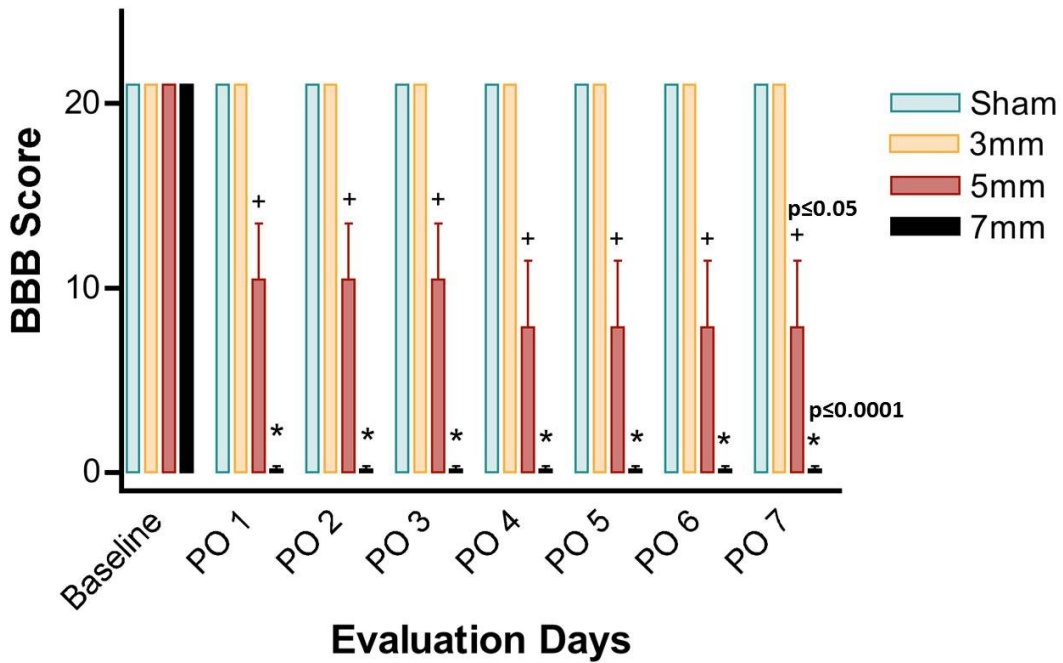
Figure 2.9 Seven days quantitative evaluation of locomotor function following a graded bidirectional distraction injury. Animals distracted 3-mm showed a normal BBB score of 21. Those distracted 5-mm showed a variable loss of motor function (0-18), while those with 7-mm lesions scored 0 or 1. $+ p \leq 0.05$ and $* p \leq 0.0001$ compared to sham/baseline. (Seifert et al. 2011)

2.3.2 Tissue Loss in the Spinal Cord is Proportional to the Degree of Spine Distraction

Histological evaluation of the distracted spinal cords confirmed the graded extent of necrotic SCI caused by the different distraction distances, as shown in Figure 2.10. Specifically, the sham and mildly distracted (3-mm) animals showed no tissue loss, as visualized by H&E staining. Moderately distracted (5-mm) animals showed an average of 2.62% tissue loss at the epicenter; whereas, severely distracted (7-mm) animals showed an average of 24.16% tissue loss at the epicenter.

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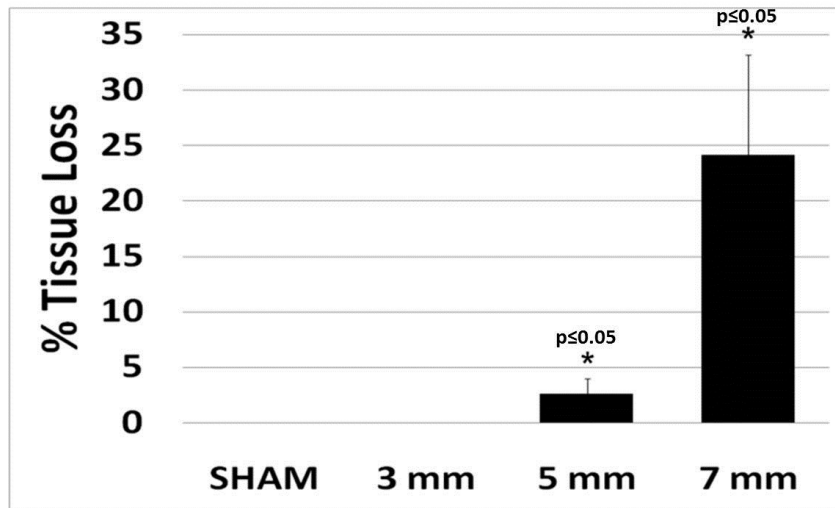
Figure 2.10 Tissue loss is proportional to the distraction length. Quantification of percent tissue loss over graded injury revealed that a 3-mm distraction does not result in significant tissue loss; however, 5-mm and 7-mm distractions both result in significant tissue loss. * $p \leq 0.05$ compared to sham. (Seifert et al. 2011)

In addition to the H&E staining, the histological evaluation of NeuN staining also revealed tissue loss that increases in proportion to the degree of spine distraction, as shown in Figure 2.11. Positive staining for GFAP and ED1 increased with distraction length, indicating that both reactive gliosis and inflammation were elicited in direct proportion to the extent of distraction injury (Figure 2.11). The up-regulation of GFAP at the injury site is similar to that observed in other models of SCI (Sofroniew 2009, Herrmann et al. 2010).
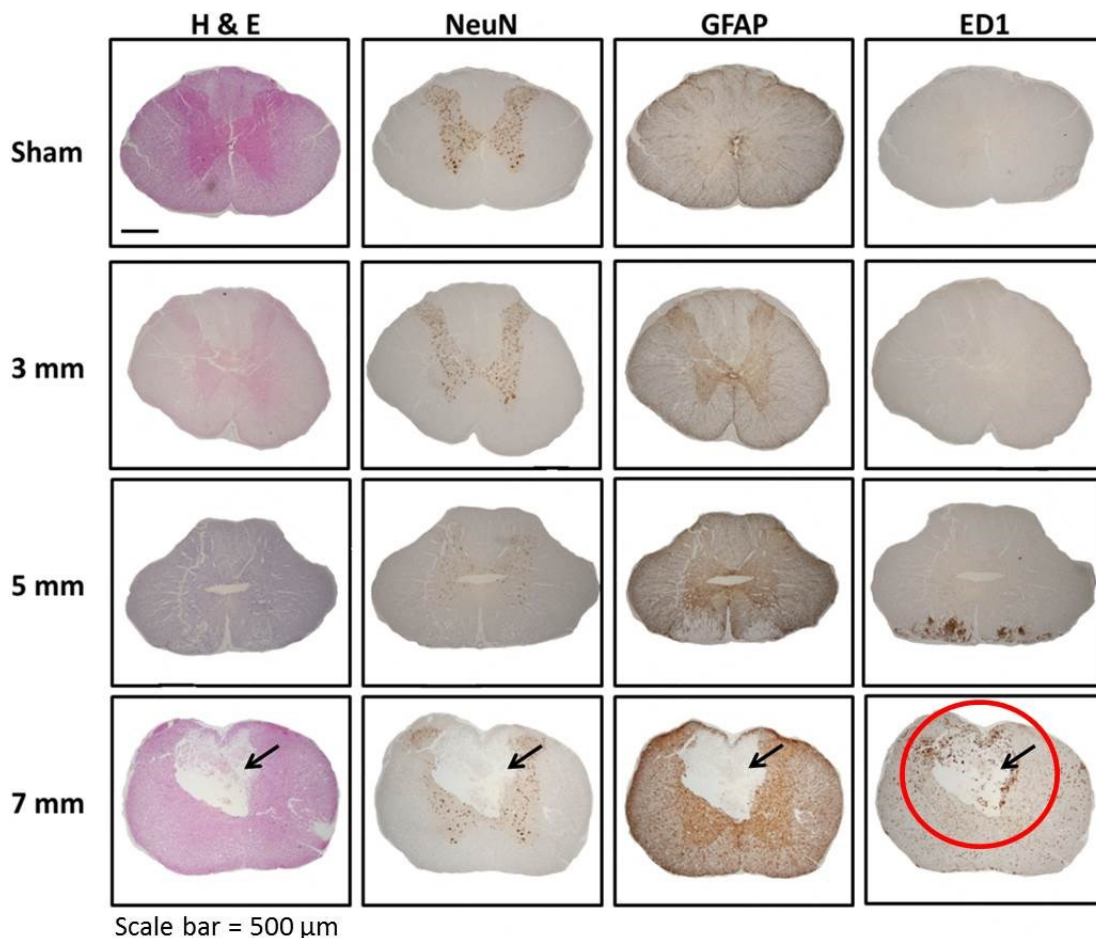
Figure 2.11 Spinal cord damage following a graded bidirectional distraction injury. Transverse spinal cord sections from sham animals and those distracted 3, 5, and 7-mm were stained for H&E, NeuN GFAP, and ED1. An increase in cell death and tissue loss (arrows) was observed in proportion to the degree of distraction length. Positive staining for GFAP and ED1 also increases with distraction length, indicating an increased number of reactive astrocytes and activated macrophages. The positive staining for ED1 in the 7-mm group (red circle) is indicative of cell debris at the injury site, confirming tissue loss in that region. Scale bar = 500 µm. (Seifert et al. 2011)

The extent of SCI produced by the 7-mm distraction was confirmed by the evident stretching of the spinal cord, which significantly reduced the diameter of the cord at the epicenter of the lesion (Figure 2.12). Specifically, gross evaluation of the distracted spinal cords revealed a highly localized injury, with a stretched epicenter exhibiting massive cell death which extended both anterior and posterior from the epicenter, affecting the dorsal columns and

sparing most of the ventral cord and white matter tracts in sections adjacent to the injury site. The massive cell death likely resulted from acute necrosis due to the excessive mechanical tissue distention (Liu et al. 1997, Galluzzi et al. 2007, Kawabata et al. 2010), followed by secondary injury events such as vascular leakage, ischemia-reperfusion, glutamate excitotoxicity and disturbances in ionic homeostasis, oxidative cell injury and inflammation that causes further apoptotic cell death (Yong et al. 1998, Sekhon and Fehlings 2001, Campos-Esparza et al. 2009).
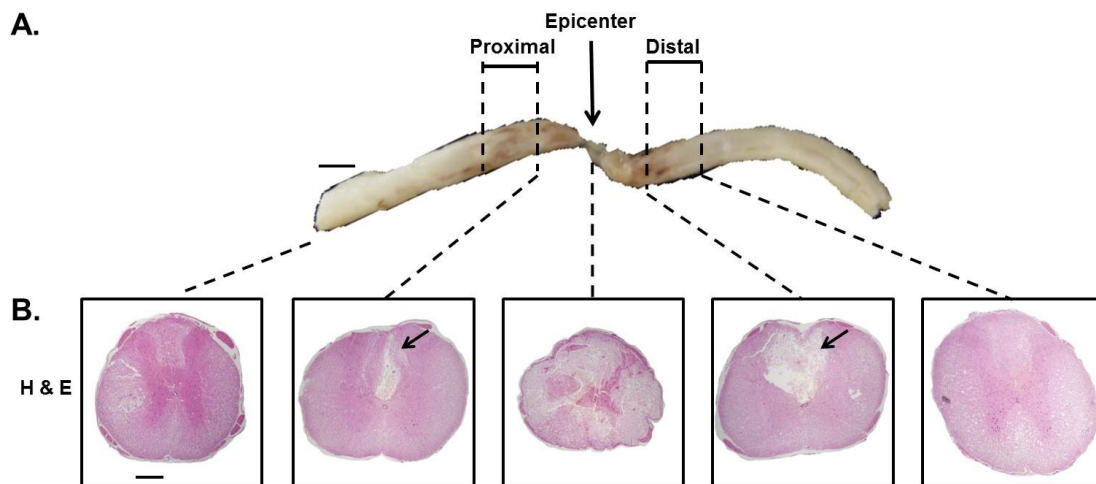
Figure 2.12 Spinal cord damage following a 7-mm bidirectional distraction injury. Gross anatomical (A) and histological (B) images of spinal cords seven days following a 7-mm distraction injury revealed that the lesion epicenter shows a dramatic reduction in tissue volume that extends to 5-mm from the lesion site following severe distraction injuries. H & E immunohistochemistry confirmed the extent of the lesion at and proximal/distal to the injury epicenter (B). Arrows indicate the areas of tissue loss. Scale bar = 3 mm (A), and 500 µm (B). (Seifert et al. 2011)

2.4 Discussion

During scoliosis corrective surgery, the most common corrective technique includes translation of tensile forces along the axis of the screws perpendicular to the intended rod direction and, thus, the application of distraction and/or compression forces (Wenger et al. 1982, Liljenqvist et al. 2001, Lee et al. 2004). A clinically relevant animal model of these forces

is necessary in order to test potential neuroprotective strategies that aim to reduce the incidence of new neurological deficits following scoliosis corrective surgery. Here we report the establishment and characterization of a novel bidirectional distraction SCI model. In contrast to the previously mentioned models of distraction, the UTA Spine Distractor can be used to apply highly controlled bidirectional distraction forces to the rat spine, as shown in Table 2.1.

Table 2.1 Comparison of Distraction Models

Model	Distraction Injury		Characterization	Advantages	Limitations
	Unidirectional	Bidirectional			
Dolan et al. 1980	X		• Electrophysiological monitoring: SERs • Spinal cord blood flow measurement		• Low injury reproducibility using manually-operated apparatus • Unidirectional injury is less clinically relevant • Lack of functional analysis
Owen et al. 1990 Naito et al. 1992 Kai et al. 1993		X	• Electrophysiological monitoring: SSEPs, NMEPs • Functional analysis: Stagnara wake-up test • Spinal cord blood flow measurement	• Bidirectional injury is more clinically relevant	• Lack of functional analysis
Dabney et al. 2004		X	• Electrophysiological monitoring: SSEPs • Functional analysis: Tarlov score	• Bidirectional injury is more clinically relevant	• Sublaminar hooks can cause unintended damage to spinal cord • Stepping motor not easily controlled • High variability following 5-mm and 7-mm distractions
Choo et al. 2007 Choo et al. 2008 Choo et al. 2009	X		• Histological analysis : H&E, fluorescein-dextran	• Linear actuators allow for increased control	• Unidirectional injury is less clinically relevant • Lack of functional analysis
Seifert et al. 2011		X	• Electrophysiological monitoring: TcMEPs • Functional analysis: BBB locomotor rating score • Histological analysis: H&E, NeuN, GFAP, ED1	• Bidirectional injury is more clinically relevant • Linear actuators allow for increased control	• Variability following 5-mm distraction

In this study, a 3-mm distraction did not produce an observable deficit in any of the outcome measures evaluated. This distance yielded results identical to those of the sham injury; although, varying some specifics of the distraction protocol may provide a mild injury at this shorter distance (variables such as speed, acceleration, time held in a distracted state, etc.).

Based on the previous study by Dabney et al. (2004), we expected that a 5-mm distraction would result in a moderate distraction injury; however, it was noted during this study that a 5-mm distraction actually resulted in dislocation, rather than a pure linear distraction, in some animals. Specifically, distracting 5-mm was sufficient to rupture the intervertebral disc, thus exposing the cord in some animals, while in others the column remained relatively intact.

Thus, the source of variability in these animals may stem from the presence or absence of spinal cord visualization during the surgical procedure. This likely explains the high variability in the 5-mm injury paradigm also reported by Dabney et al. (2004).

The electrophysiological, histological and behavioral data strongly suggest that a 7-mm distraction in the rat is unnecessarily drastic for modeling the axial pullout of the spine during surgical correction of scoliosis. A distraction of 7-mm created a vertebral separation equivalent to two vertebral segments in the rat (Porter 2000, Flynn and Bolton 2007). Since the human thoracic spine contains 12 vertebral segments and averages 28 cm in length (Panjabi et al. 1991, Liljenqvist et al. 2002, Sucato and Duchene 2003), if extrapolated, such separation approximates 46 mm in adult humans, a defect which will require forces normally not applied during scoliosis distraction and spine instrumentation and which is unlikely to occur during surgery. The severity of the 7-mm distraction in the rat better resembles shear fracture-dislocations of the thoracic and lumbar spine associated with forceful hyperextension (i.e., Lumberjack Paraplegia) (Denis and Burkus 1992, Erb et al. 1995).

Despite the improved characteristics of the UTA Spine Distractor compared to other available models, the current injury paradigm has several limitations with respect to clinical scoliosis surgery: 1) Unlike spine deformity correction surgery that can last between 4 and 16 hours depending on the severity of the case, the induction of injury in current animal models of spine distraction requires only seconds (approximately 3.5 seconds for the UTA Spine Distractor). Thus, the rapid injury induction described in this study does not accurately model the forces typically imparted to the cord during spine deformity surgery. 2) Surgeons constantly monitor TcMEPs throughout the process of correction; however, the fast distraction in current animal models obligates the recording of TcMEPs before and immediately after but not during distraction. While the acquisition of TcMEP amplitude changes at the exact onset of distraction would provide very useful data, the feasibility is a challenge in the current setup. 3) Scoliosis presents spine deformities that involve lateral deviation and rotation of vertebral bodies. Since

rodents with scoliotic spines are not available, our model cannot account for SCI due to rotation or realignment from lateral deviation; this model only mimics the stretching/distraction forces used to help in spine realignment. Overcoming these limitations will establish a more complete animal model for spine deformity correction.

In 1971, it was postulated that the two likely contributions to neurologic deficit following the surgical correction of scoliosis are mechanical damage to the spinal cord tissue and/or mechanical damage to the vasculature of the spinal cord, as illustrated in Figure 2.13 (Keim and Hilal 1971).
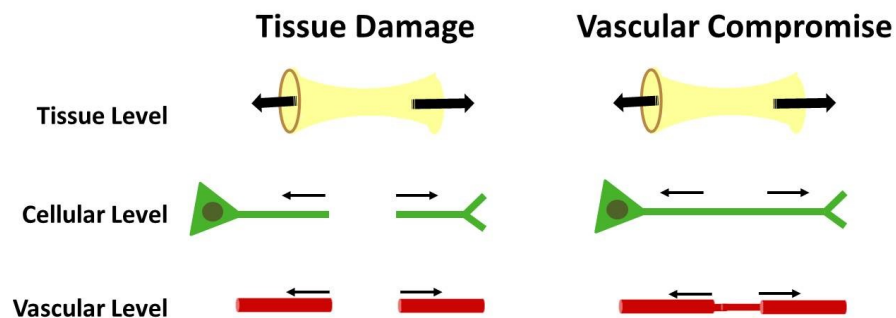
Figure 2.13 Schematic comparing the effects of tissue damage with those of vascular compromise following SCI.

In the clinic, there is a lack of obvious mechanical damage to the spinal cord tissue (hemorrhage, edema, and trauma) in the absence of observed hardware interference (personal communication with Daniel J. Sucato, M.D., M.S. at Texas Scottish Rite Hospital for Children in Dallas, TX). Compared to the massive mechanical damage to the spinal cord tissue observed in this study, the damage observed in the clinic is more subtle, which suggests the onset of vascular compromise; therefore, we hypothesized that the functional deficits observed in the clinic are primarily a result of vascular compromise. Further modifications of the distraction device and injury paradigm are needed in order to test this hypothesis.

2.5 Conclusion

Together, the results of this study demonstrate that the UTA Spine Distractor can be used to apply highly controlled bidirectional distraction forces to the rat spine. This highly reproducible spine distraction and consequent SCI will contribute significantly as a test platform for the future development of neuroprotective therapies that can eventually minimize or eliminate the development of new neurological deficits in patients undergoing corrective spine surgery. In this study, the 7-mm distraction paradigm was found to be too severe for modeling the flexion/distraction injuries that can occur in the clinic. There is evidence that indicates a vascular component to the onset of SCI in the clinic during spine deformity surgery, yet there are no models to mimic this. Therefore, further modifications to the 5-mm distraction paradigm will likely result in a model that better mimics the neurological deficits that can result from the application of distractive forces during spine deformity surgery.

CHAPTER 3

ISOLATION OF VASCULAR COMPROMISE FROM TISSUE DAMAGE FURTHERS THE
ELUCIDATION OF INJURY MECHANISMS INVOLVED IN DISTRACTION SPINAL CORD
INJURY

3.1 Introduction

The nature of SCI is complex, characterized by a primary mechanical insult to the
tissue and/or vasculature followed by the activation of secondary signaling cascades that lead
to glutamate excitotoxicity, inflammation, and oxidative stress (Demopoulos et al. 1979, Choi et
al. 1987, Lipton and Rosenberg 1994). These various secondary injury mechanisms often
culminate in the death of sensitive neurons, resulting in neurological deficits.

In the clinic, IONM is aimed at early detection of possible complications to allow for
surgical intervention to mitigate unintended damage to the spinal cord (Gonzalez et al. 2009,
Malhotra and Shaffrey 2010). However, one study recently identified a 59% complication rate
associated with VCR for the correction of severe spine deformity in which 27% of patients had
either an IONM change or a failed wake-up test due to spinal cord or nerve root deficit while no
cases resulted in permanent paralysis (Lenke et al. 2013). In the absence of observed hardware
interference, the occurrence of SCI during spine surgery is hypothesized to result, in part, from
direct spinal cord stretching (Mcafee and Bohlman 1985, Vitale et al. 2010, Master et al. 2011);
however, it remains unknown whether the primary injury following distraction is predominantly
mechanical damage to the tissue or mechanical damage to the vasculature.

Despite the incidence of distraction injury, the majority of studies investigating the
underlying nature of SCI utilize animal models of transection or contusion injury (Ek et al. 2012,
Costa et al. 2013, Murphy et al. 2013, Streijger et al. 2013). A few previous models of
distraction have sought to characterize the injury, including effects on SCBF; however,
deficiencies in the models, as well as a lack of functional assessment, have limited their ability

to effectively elucidate the injury mechanisms and be used reliably for future screening of neuroprotective strategies (Dolan et al. 1980, Naito et al. 1992, Dabney et al. 2004, Choo et al. 2007, Choo et al. 2008, Choo et al. 2009). To overcome these limitations, we previously fabricated the UTA Spine Distractor to study the acute effects of a graded distraction injury (Seifert et al. 2011). Similar to Dabney et al. (2004), we observed a graded acute injury response following 3, 5 and 7-mm distractions. Specifically, a 7-mm distraction resulted in massive, mechanical tissue damage and was, thus, too severe for modeling the flexion/distraction injuries that can occur in the clinic. Since the nature of the primary injury mechanism following distraction SCI remains unknown, the need for a model that would allow for distinction between tissue damage and vascular compromise remains.

Here we report our findings aimed at elucidating the injury mechanisms involved in distraction SCI using a modified 5-mm distraction injury paradigm. In order to reduce the variability associated with a 5-mm distraction, we reduced the speed of distraction from 1 mm/sec to 0.5 mm/sec and fabricated a modified clamping mechanism. In addition to modifying parameters associated with the distraction device, we held each animal in a distracted position for 15 minutes before clamp release in order to better model the distractive forces typically imparted to the spinal cord during spine deformity surgery (i.e., extended period of traction to aid in the alignment of the spine). Since previous studies have shown that a 5-mm distraction is expected to produce a moderate injury (Dabney et al. 2004, Seifert et al. 2011), we hypothesized that a prolonged 5-mm spinal distraction, in addition to the known mechanical forces, will introduce a hypoxic insult to the spinal cord that results in a recordable reduction in oxygenation levels in the distal cord parenchyma. To test this hypothesis, we directly measured the partial pressure of oxygen (pO_2) before, during, and after injury in the spinal cord segments distal to the injury site to determine the extent to which distraction SCI induces low oxygen levels within the spinal cord.

We also hypothesized that the mechanical disruption and period of low oxygenation following distraction injury is sufficient to negatively affect mitochondrial function, leading to an increase in ROS formation and protein oxidation. To test this hypothesis, we examined tissue homogenates from spinal cord sections obtained at various time points after distraction injury for levels of ROS and protein carbonyls (protein oxidation).

<u>3.2 Materials and Methods</u>

3.2.1 Modified Clamping Mechanism for UTA Spine Distractor

During our previous study, it was observed that the UTA Spine Distractor clamps rotated upward as the force of distraction was applied, resulting in dislocation in some animals rather than a pure linear distraction, as shown in Figure 3.1.
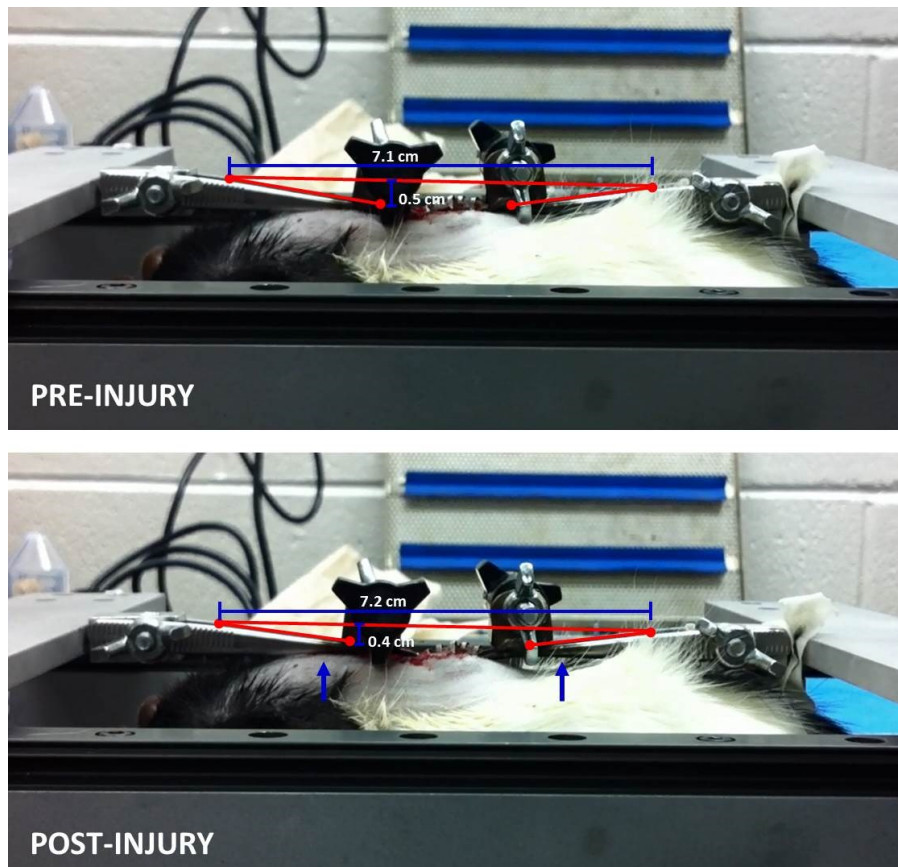
Figure 3.1 Illustration of clamp position pre- and post-injury using original clamping mechanism. The clamps rotate upward (blue arrows) as the force of distraction is applied such that red lines positioned along the top of each clamp are more parallel to the device post-injury.

Therefore, a modified clamping mechanism was fabricated and adapted to fit onto the UTA Spine Distractor to increase control and decrease the variability associated with a 5-mm distraction. The modified clamping mechanism includes an adjustable rotary pivot feature and two vertical ball slide assemblies for easy positioning and increased vertical travel of the clamps, as well as a horizontal ball slide assembly for incorporation of the 50 lb. capacity load cell (MFM- 050-200-A*C03, Loadstar Sensors, Fremont, CA) for force measurement (Figure 3.2).
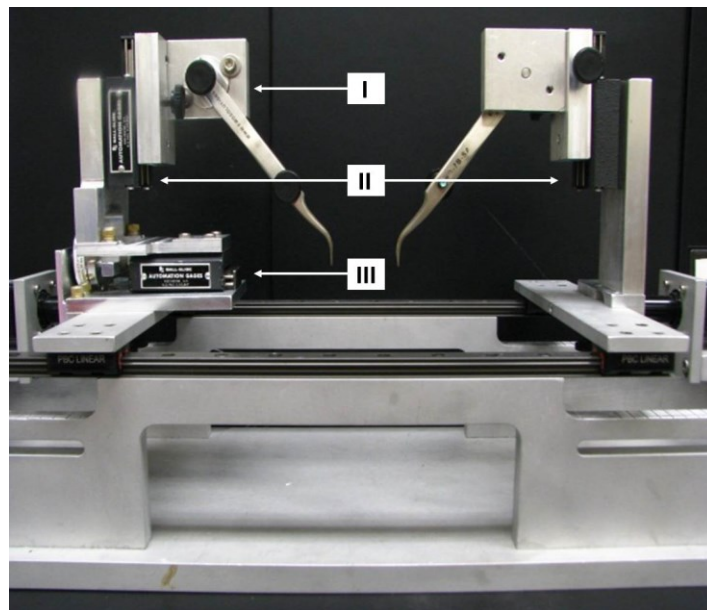
Figure 3.2 Modified clamping mechanism involving the use of an adjustable rotary pivot feature (I), two vertical ball slide assemblies (II), and a horizontal ball slide assembly (III).

The modified clamping mechanism was designed to overcome the primary limitation of the original setup. Specifically, after the clamps in the original clamping mechanism are placed around the vertebral bodies, the point of contact of the clamps with the vertebrae is lower than the axis of pull, resulting in a combined bending moment as the force of distraction is applied. Because the joint at which the clamps are attached to the device is non-rigid, the effect of this combined bending moment is an upward rotation of the clamps as the force of distraction is applied (Figure 3.3A). In contrast, the clamps in the new clamping mechanism, comprised of

modified curved, serrated tweezers (EWD-7B-SA, EWD Solutions, Carrollton, TX), interface with the vertebral bodies in the same manner as the previous design but are more precisely constrained to prevent upward rotation during distraction in order to ensure that the force of distraction is exerted as pure tension along the horizontal axis of the spinal column (Figure 3.3B).
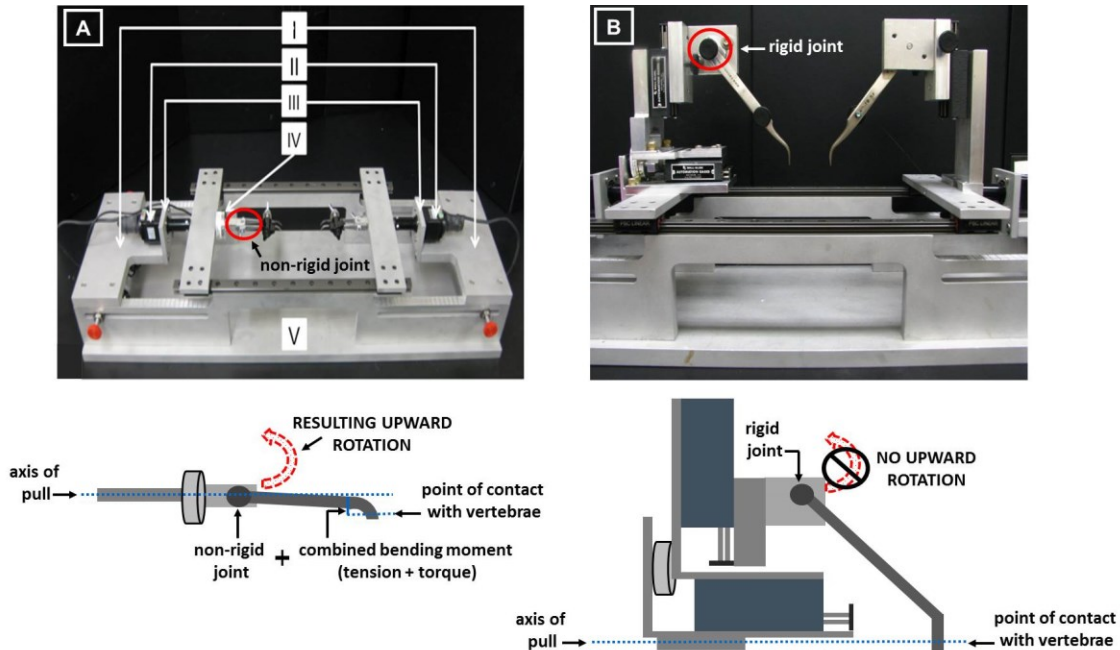
Figure 3.3 Comparison of the clamp attachment in the original clamping mechanism (A) with the modified clamping mechanism (B) on the UTA Spine Distractor.

There are two additional limitations of the original clamping mechanism that the new design overcomes. First, the tips of the clamps in the original clamping mechanism are not perpendicular to the spinal column when placed around the vertebral bodies (Figure 3.4A). Depending on exactly how the tips of the clamps contact the vertebrae, this positioning can exacerbate the combined bending moment. In the new design, the adjustable rotary pivot feature enables exact positioning of the clamp tips such that they are perpendicular to the spinal column and the force of distraction is exerted as pure tension across the spine (Figure 3.4B).

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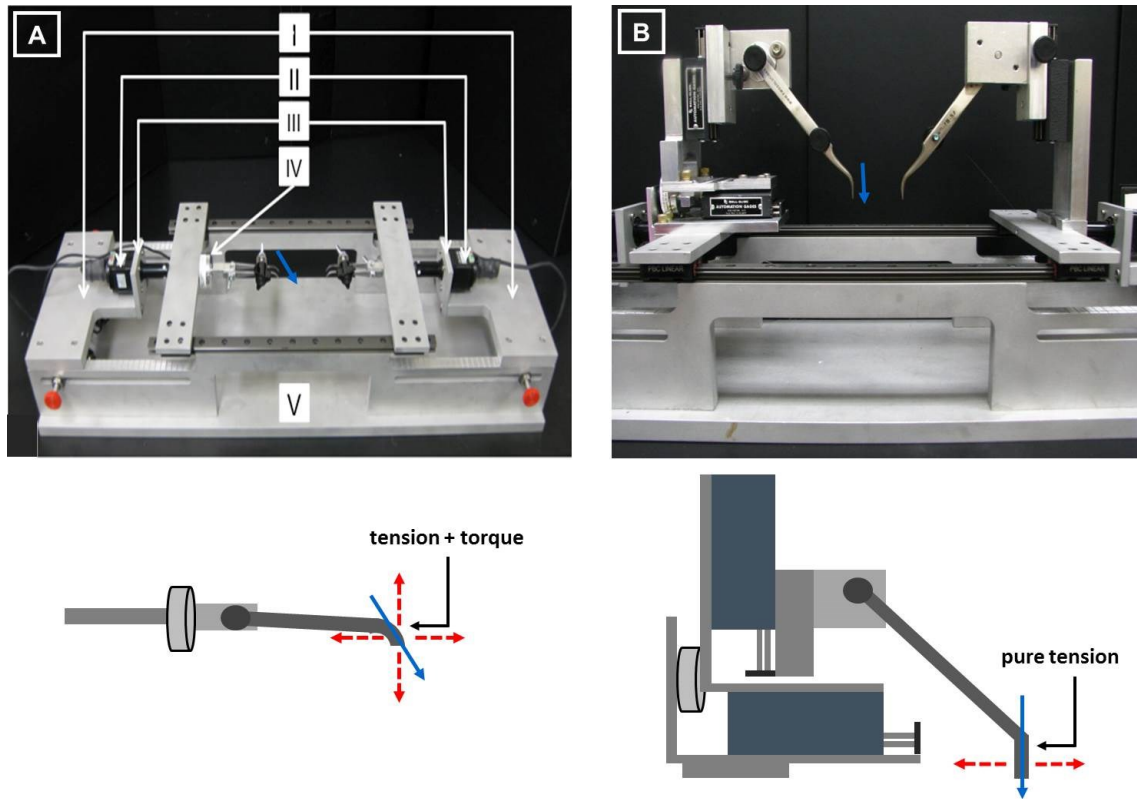
Figure 3.4 Comparison of the angle of the clamps in the original clamping mechanism (A) with the modified clamping mechanism (B) on the UTA Spine Distractor.

In addition, the load cell, in its original position, is connected to the non-rigid joint and, thus, is subject to the combined bending moment, potentially resulting in an inaccurate force measurement (Figure 3.5A). In the new design, the load cell is positioned such that it only measures the force across the top and bottom of the horizontal ball slide and, thus, only the force of pure tension across the spine (Figure 3.5B).
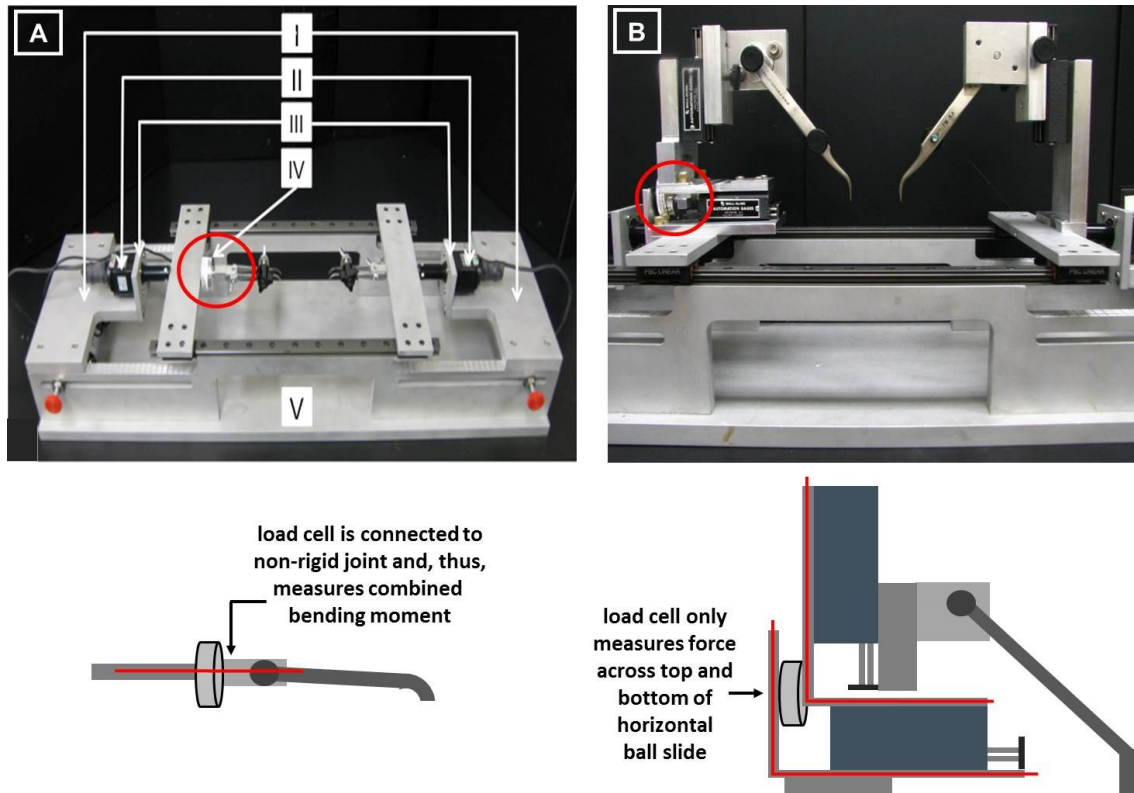
Figure 3.5 Comparison of the load cell placement in the original clamping mechanism (A) with the modified clamping mechanism (B) on the UTA Spine Distractor.

3.2.2 Experimental Design for Specific Aim 2.1

The second specific aim was directed at evaluating the sub-acute secondary effects of a clinically relevant distraction injury. We first characterized the acute injury response to a prolonged bidirectional distraction SCI, as shown in Figure 3.6.
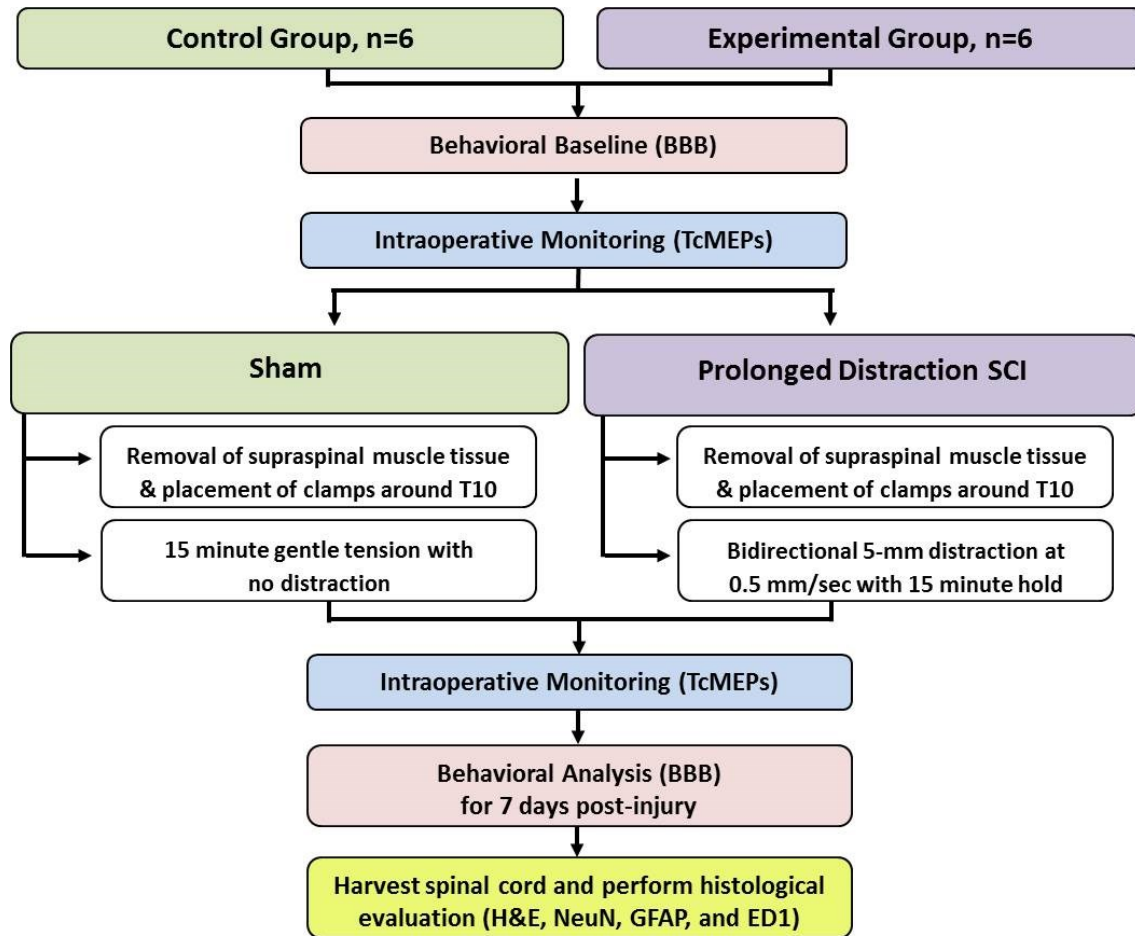
Figure 3.6 Experimental design for Specific Aim 2.1.

3.2.3 Distraction Spinal Cord Injury of Adult Rats

Twelve female Long-Evans rats (275-325 g) were divided into two groups and used for characterization of the acute injury response to a prolonged distraction injury: 5-mm distraction + 15 min hold (n = 6) and sham + 15 min gentle tension (negative control, n = 6). All surgical procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of The University of Texas at Arlington. As previously described (Seifert et al. 2011), following anesthetization with sodium pentobarbital (50 mg/kg, intraperitoneal), the animal's back was shaved and cleaned, and the vertebral column was exposed by dissection of the paraspinous muscles. The vertebral clamps were placed around the lateral processes

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caudal to T9 and rostral to T11, and gentle tension was applied prior to distraction (2.48 +/- 0.88 N). In contrast to our previous study, a 5-mm distraction injury was induced at 0.5 mm/sec, and the animal was held in the distracted position for 15 minutes after which the animal was removed from the clamps and the muscle layer and skin were closed. To serve as a negative control, six animals received a sham injury in which clamp placement was followed by the application of gentle tension for 15 minutes. Antibiotic (cephazolin; 5mg/kg, intramuscular) and pain medication (buprenorphine; 0.05–0.1 mg/kg, subcutaneous) were administered for three days post-injury.

3.2.4 Electrophysiological Monitoring

A Cadwell Cascade™ intraoperative monitoring system was used to monitor TcMEPs before and after injury, as previously described (Seifert et al. 2011). Intraoperative recordings were taken after placement in the clamps and gentle tension but before distraction to ensure the spinal cord was intact prior to injury. In addition to the reference and ground electrodes, two stimulating electrodes were placed over the motor cortex, and recording electrodes were placed in the right and left deltoid (proximal to the injury site, negative controls) and gluteal (distal to the injury site) muscles. A train of three stimulation pulses (8-10 V amplitude, 50-µs duration, 2-ms interstimulus interval) was applied over the motor cortex, and TcMEP signals were recorded, filtered, amplified, processed and stored. Percent amplitude decrease was determined by comparing post-injury amplitudes to baseline values.

3.2.5 Behavioral Analysis

3.2.5.1 Dynamic Plantar Aesthesiometer

Mechanoception was evaluated using a Dynamic Plantar Aesthesiometer (Ugo Basile, VA, Italy) to measure the force threshold required to elicit paw withdrawal following mechanical stimulation. All animals were acclimated to the testing apparatus, consisting of a three-compartmental, plastic enclosure on top of a wire mesh platform, for 15 minutes prior to testing. Testing involved stimulation of the plantar surface of each hind paw using a metal filament

connected to a moveable touch stimulator. The stimulator was programmed to apply 50 g of force over 10 seconds until paw withdrawal was elicited. During the testing period, each hind paw was stimulated three times in 5-minute intervals, and the resulting force values were averaged to obtain the force threshold for that testing period. Baseline force threshold was determined by averaging the force threshold over three days prior to injury. Post-injury testing occurred daily for seven days, and sensory deficit was determined by comparing post-injury force threshold to baseline values.

3.2.5.2 BBB Locomotor Rating Score

Gait analysis was performed using the 22-point Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale (Basso et al. 1995, Basso et al. 1996). As described previously (Seifert et al. 2011), hindlimb function was assessed daily by two observers blinded to the groups for seven days following injury, and functional deficit was determined by comparing post-injury BBB scores to behavioral baselines established prior to injury.

3.2.6 Histological Analysis

Following transcardial perfusion with 4% paraformaldehyde, spinal cords were harvested, fixed overnight, and processed for paraffin embedding. A 15-mm segment containing T9-T11 was isolated and divided into three 5-mm segments corresponding to proximal, injury epicenter, and distal sections. Transverse sections were cut and stained with hematoxylin and eosin (H&E) to evaluate tissue loss. As described previously (Seifert et al. 2011), immunohistochemistry was used to visualize neuronal bodies (NeuN; 1:200 Mouse anti-NeuN; Millipore, Billerica, MA), as well as reactive astrocytes and activated macrophages labeled with antibodies against glial fibrillary acidic protein (GFAP; 1:1000 Mouse anti-GFAP; Millipore, Billerica, MA) and ectodermal dysplasia 1 (ED1; 1:100 Mouse anti-CD68; Millipore, Billerica, MA), respectively. Briefly, deparaffinized sections were blocked with endogenous peroxidase with 3% H_2O_2 for 10 minutes, rinsed, then blocked in universal blocking solution (Biogenex; San Ramon, CA) for 10 minutes. Sections were incubated in primary antibody overnight at 4°C,

rinsed with tris-buffered saline, then incubated with HRP-conjugated secondary antibodies for one hour at room temperature (1:500; Millipore, Billerica, MA). Immunodetection was visualized by DAB (Dako, Carpinteria, CA).

The intensity distribution along a straight line across each H&E-stained spinal cord section was quantified using the profile tool in the Zeiss LSM510 software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Briefly, a straight line was positioned horizontally through laminas 4 and 5 of the dorsal horns, posterior to the dorsal corticospinal tract, in order to allow for comparison to the massive tissue loss observed in the same region in the H&E-stained spinal cord sections from animals distracted 7-mm in the previous study (positive control), as shown in Figure 3.7. The signal intensities along the straight line were then averaged to obtain the average signal intensity across the section. Using this method, regions of tissue loss show higher signal intensity.
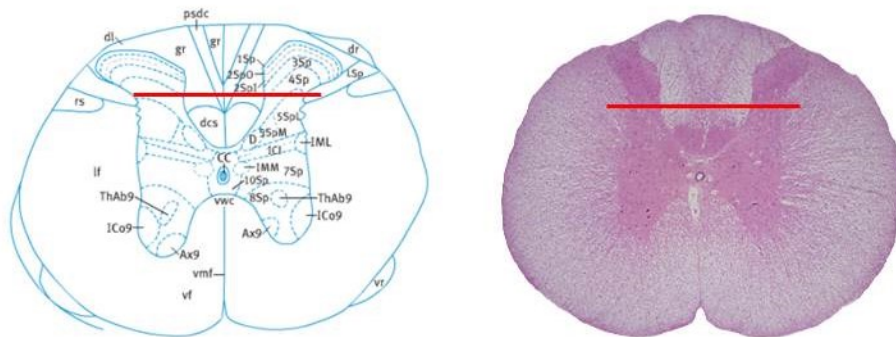
Figure 3.7 Diagram of straight line positioned across H&E-stained spinal cord sections for quantification of intensity distribution. Specifically, the straight line was positioned horizontally through laminas 4 and 5 of the dorsal horns, posterior to the dorsal corticospinal tract. Image on left adapted from (Watson et al. 2009).

3.2.7 Experimental Design for Specific Aim 2.2

The second specific aim was directed at evaluating the sub-acute secondary effects of a clinically relevant distraction injury by determining the level of hemodynamic instability. Following characterization of the acute injury response to a prolonged bidirectional distraction SCI, we monitored the partial pressure of oxygen (pO_2) before, during, and after injury in the

spinal cord segments distal to the injury site to determine the extent to which distraction SCI induces low oxygen levels within the spinal cord, as shown in Figure 3.8.
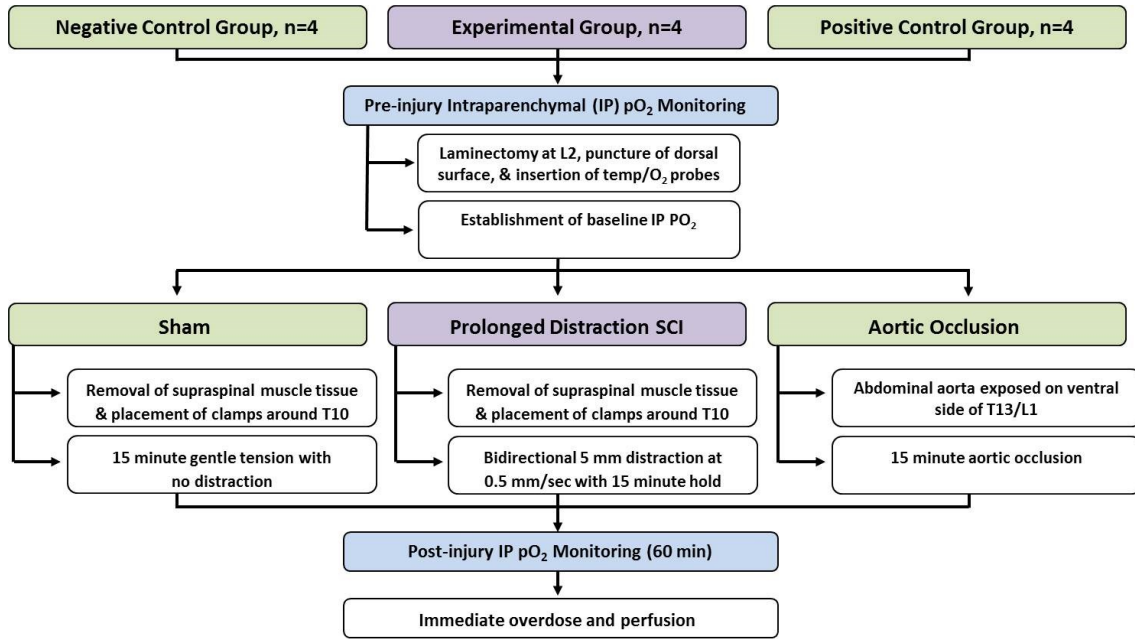
Figure 3.8 Experimental design for Specific Aim 2.2.

3.2.8 Intraparenchymal pO₂ Monitoring

Twelve female Long-Evans rats (275-325g) were used for determination of changes in intraparenchymal (IP) pO_2 levels in response to a prolonged distraction injury. Animals were divided into three groups: 5-mm distraction + 15 min hold (n = 4), sham + 15 min gentle tension (negative control, n = 4), and 15 min abdominal aortic occlusion (positive control, n = 4). All animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). For IP pO_2 monitoring in animals undergoing a sham or 15 minute prolonged 5-mm distraction injury, a laminectomy was performed at L2, and a 0.8 mm diameter Licox C8.B temperature electrode (Integra-LS, Plainsboro, NJ) was inserted caudally and advanced approximately 2 cm alongside the spinal cord within the spinal column for tissue temperature detection. The dorsal surface was then punctured at the dorsal sulcus of the spinal cord using a 25-gauge needle to allow for

insertion of a 0.5 mm diameter Licox CC1.R oxygen electrode (Integra-LS, Plainsboro, NJ) rostrally into the parenchymal space (Figure 3.9). The oxygen probe was advanced to the level of T12 (approximately 2 cm) to record just distally to the injury, and baseline IP pO_2 was measured prior to injury.

A.

B.
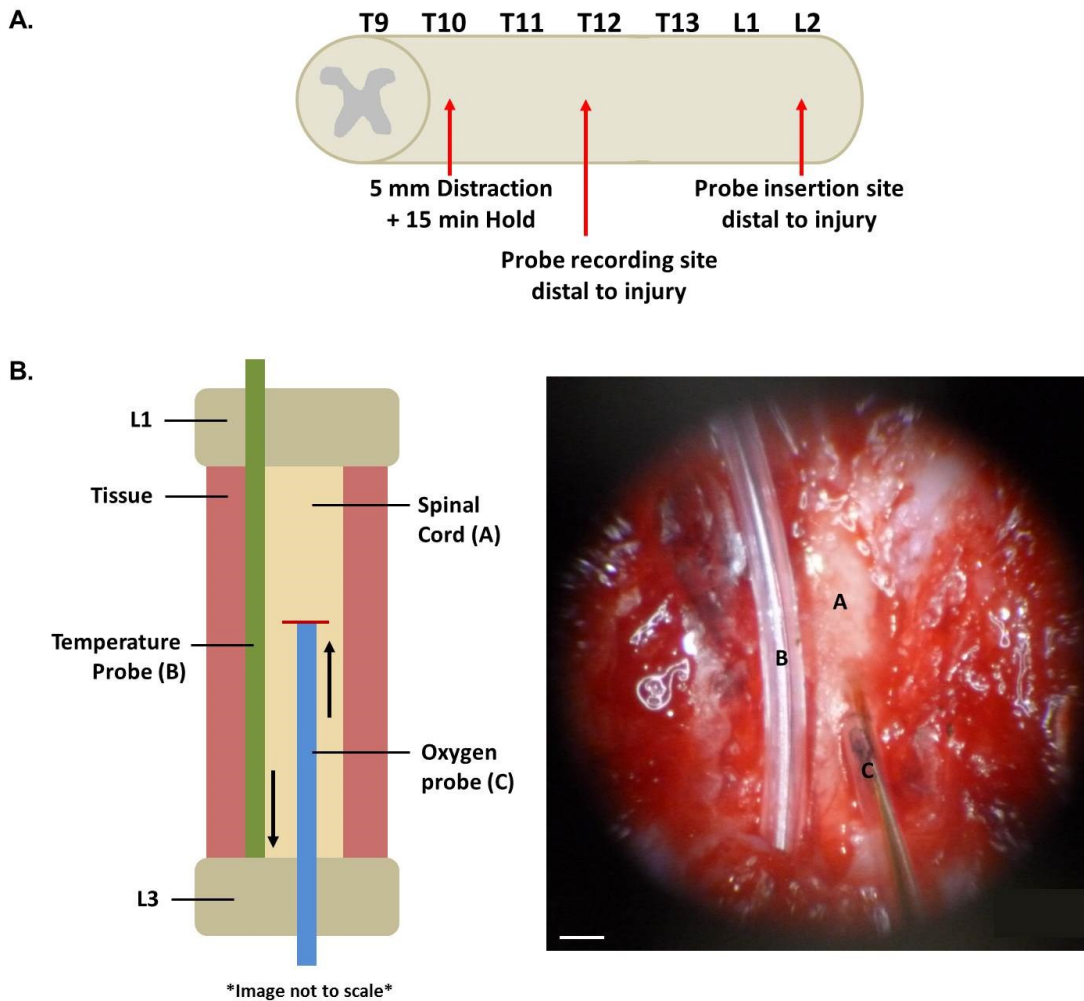
Figure 3.9 Setup for IP pO_2 monitoring in animals undergoing sham injury and prolonged distraction. (A) Diagram of oxygen probe placement. Following a laminectomy at L2, the probe is inserted rostrally and advanced to the level of T12 (approximately 2 cm). (B) Diagram and visualization of temperature and oxygen probe insertion in spinal cord. Scale bar = 0.8 mm.

In animals undergoing a 15 minute abdominal aortic occlusion, following a laminectomy at L2, the temperature electrode was inserted rostrally and advanced approximately 2 cm alongside

the spinal cord within the spinal column. The oxygen electrode was then inserted caudally and advanced approximately 5 cm into the parenchymal space to allow for immediate detection of the occlusion (Figure 3.10). An incision was made in the abdominal cavity, and the abdominal aorta was exposed on the ventral side of the T13 and L1 vertebrae and occluded for 15 minutes. Following each injury, the oxygen probe was inspected to ensure no displacement, and post-injury values were recorded for 60 minutes. All animals were immediately sacrificed with sodium pentobarbital (120 mg/kg; intraperitoneal) following each procedure.
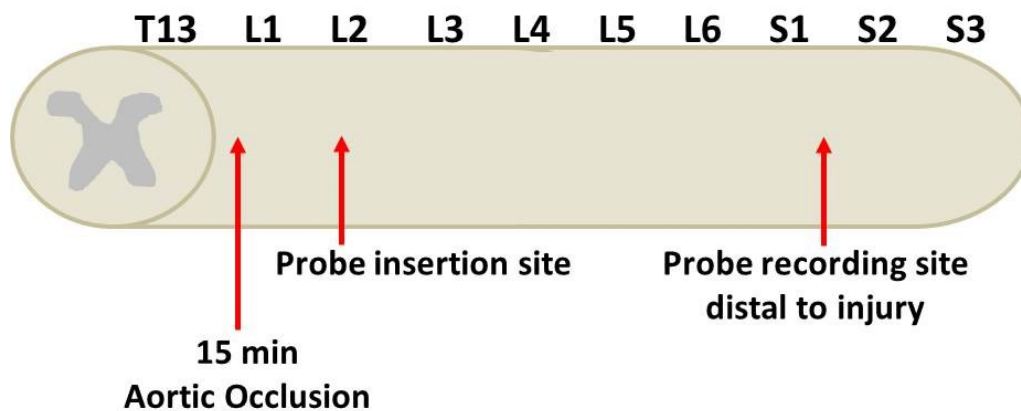
Figure 3.10 Diagram of oxygen probe placement in animals undergoing aortic occlusion. Following a laminectomy at L2, the probe is inserted caudally and advanced approximately 5 cm.

3.2.9 Experimental Design for Specific Aim 3

The third specific aim was directed at determining the contribution of secondary injury mechanisms, particularly the level of oxidative stress, to the injury. Specifically, we examined tissue homogenates from spinal cord sections obtained at various time points after distraction injury for levels of ROS formation and protein carbonyls (protein oxidation), as shown in Figure 3.11.
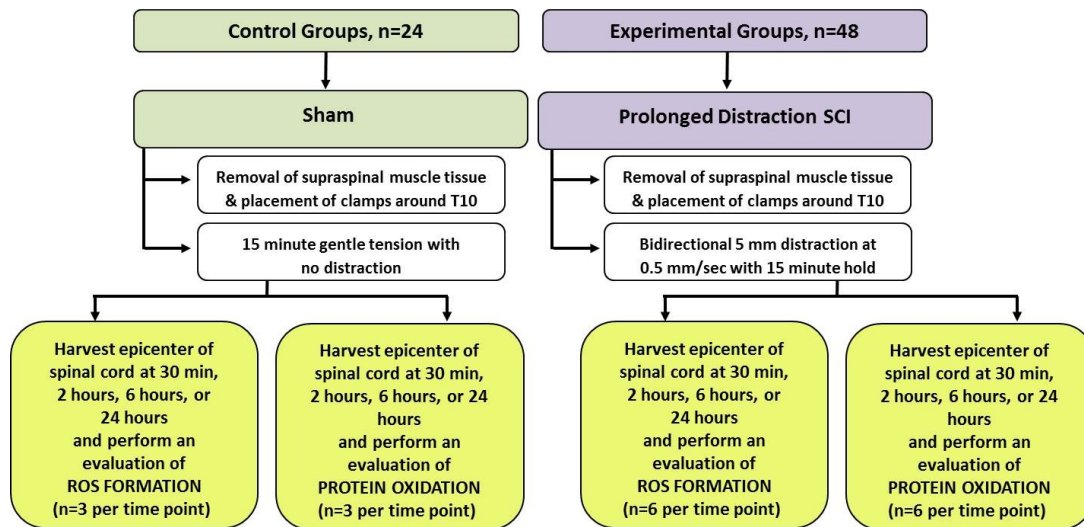
Figure 3.11 Experimental design for Specific Aim 3.

3.2.10 Evaluation of Reactive Oxygen Species and Protein Oxidation

Thirty-six female Long-Evans rats (275-325g) were used for evaluation of reactive oxygen species (ROS) and protein oxidation following prolonged distraction injury. Following either a 15 minute prolonged 5-mm distraction injury (n=6 per time point) or a sham injury (negative control, n=3 per time point), T10 spinal cord sections weighing approximately 200-300 mg were harvested at four post-injury time points (30 minutes, 2 hours, 6 hours, and 24 hours). Each tissue section was rinsed, homogenized in 50 mM phosphate buffer, pH 6.7, containing 1 mM EDTA, and centrifuged. The supernatant was divided into two microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80ºC. Once all samples were collected, free radical levels were determined using the OxiSelect In Vitro ROS/RNS Assay Kit (STA-347, Cell Biolabs, Inc., San Diego, CA). Fluorescence was measured at 480 nm excitation / 530 nm emission. A hydrogen peroxide standard (20 µM) was used as a positive control. All samples were run in duplicate on a 96-well plate. Protein carbonyl content, an indicator of protein oxidation, was determined using the Protein Carbonyl Assay Kit (10005020, Cayman Chemical Company, Ann Arbor, MI). Absorbance readings were measured between 360 and 385 nm and averaged. All samples were run in duplicate on a 96-well plate.

3.2.11 Statistical Analysis

TcMEP amplitude reduction was statistically analyzed using a one-way ANOVA on the Ranks followed by Bonferroni's post hoc test. Response to mechanical stimulation, BBB score, pO_2 decrease over time, ROS generation, and protein oxidation were analyzed using Repeated Measures Analysis followed by a one-way ANOVA or one-way ANOVA on the Ranks to compare groups at each time point. Quantification of average signal intensity in H&E sections was analyzed using a one-way ANOVA followed by Bonferroni's post hoc test. An α level ≤ 0.05 was considered significant.

<div align="center">

3.3 Results

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3.3.1 Prolonged Distraction Results in Mild Functional Deficit with No Observable Tissue Loss

Interestingly, electrophysiological monitoring revealed that TcMEP amplitudes recorded both proximally (data not shown) and distally to the injury site are not significantly affected by a 15 minute prolonged 5-mm distraction injury, as seen by the representative traces shown in Figure 3.12A. Quantification confirmed that the average percent TcMEP amplitude decreases recorded distally to the injury site following sham and 15 minute prolonged distraction injuries are not significantly different (Figure 3.12B). In some animals, the TcMEP amplitude was actually increased post-injury, most likely due to the level of anesthesia, in addition to variability among animals.
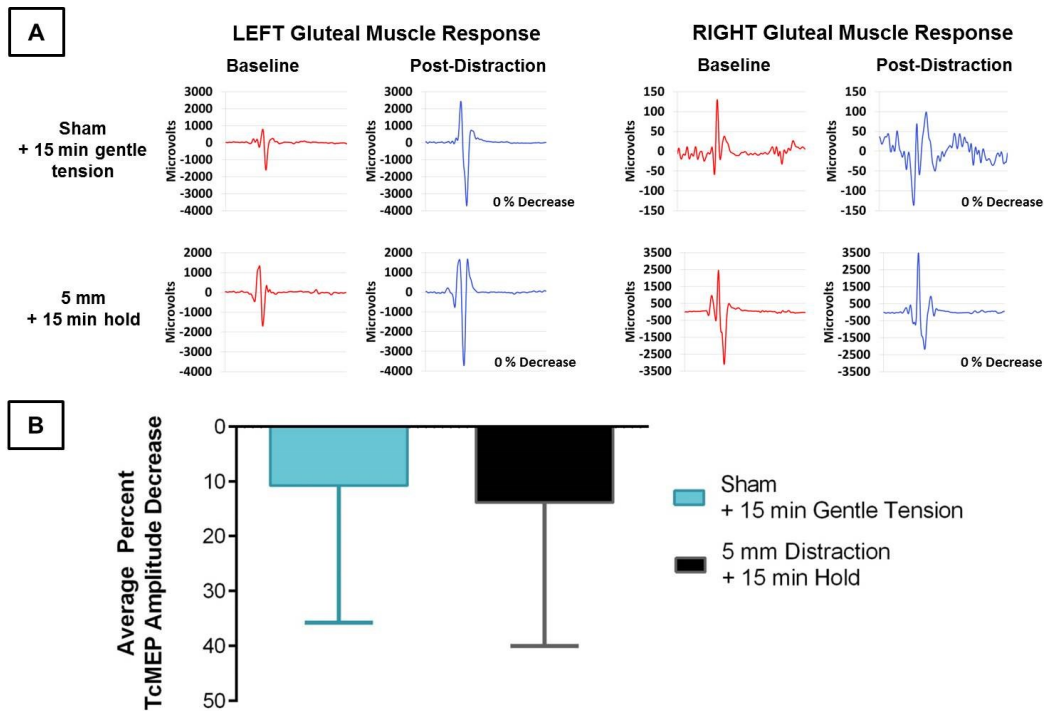
Figure 3.12 TcMEP amplitude is not significantly reduced following 15 minute prolonged distraction. (A) Representative TcMEP recordings distal to the injury site. (B) Quantification of average percent TcMEP amplitude decrease revealed that motor-evoked potentials recorded distal to the injury site are not significantly affected following a 15 minute prolonged 5-mm distraction injury. Data represent the average and standard deviation (n = 6 per sham and 15 min prolonged distraction groups).

While TcMEP amplitude was not significantly affected following injury, behavioral assessment revealed a highly reproducible, mild motor deficit following prolonged distraction. As shown in Figure 3.13A, quantification of response to mechanical stimulation showed that a 15 minute prolonged distraction injury does not significantly affect mechanoceptive response for one week compared to sham/baseline. However, quantification of BBB locomotor rating score confirmed that a 15 minute prolonged distraction does result in a consistent mild motor deficit for one week post-injury compared to sham/baseline, as shown in Figure 3.13B. Specifically, none of the animals that underwent a sham injury exhibited hindlimb rotation post-injury and remained at a BBB score of 21 for one week following injury. In contrast, all animals that underwent a 15 minute prolonged distraction exhibited hindlimb rotation and, thus, had a BBB

score of 18 immediately following injury which remained constant for the seven-day evaluation

period. The lack of variability in the 15 minute prolonged distraction group is due to the fact that

the BBB locomotor rating score does not account for the frequency of hindlimb rotation.
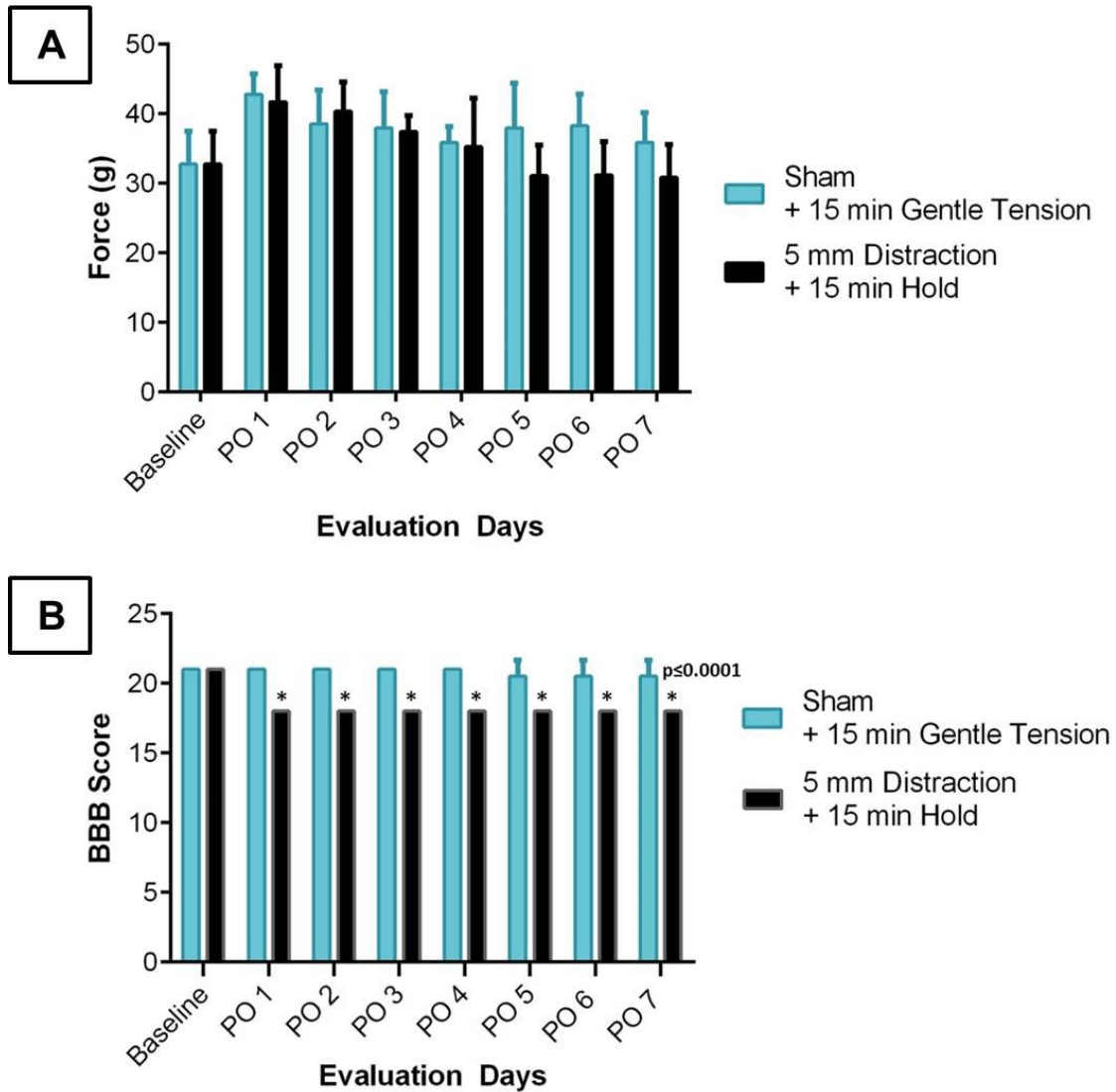
Figure 3.13 Motor function is impaired following 15 minute prolonged distraction. (A)
Mechanoceptive response is not significantly affected following a 15 minute prolonged
distraction. (B) Evaluation of BBB locomotor rating score over seven days following a 15 minute
prolonged 5-mm distraction revealed a significant, highly reproducible mild motor deficit.
Specifically, all animals that underwent a 15 minute prolonged distraction exhibited hindlimb
rotation and, thus, had a BBB score of 18 for one week post-injury. Data represent the average
and standard deviation (n = 6 per sham and 15 min prolonged distraction groups). *p≤0.0001
compared to sham/baseline.

Consistent with the observed mild functional deficit, histological analysis revealed that the structural integrity of the spinal cord was not compromised following a 15 minute prolonged distraction. Specifically, no tissue loss was detected in the H&E-stained spinal cord sections (Figure 3.14). In addition, neuron-depleted regions (indicated by NeuN) and positive staining for GFAP and ED1 were not visualized in the spinal cord following injury (Figure 3.14). These results indicate that reactive gliosis and macrophage activation, known effectors of secondary injury cascades, are absent in this mild injury paradigm.
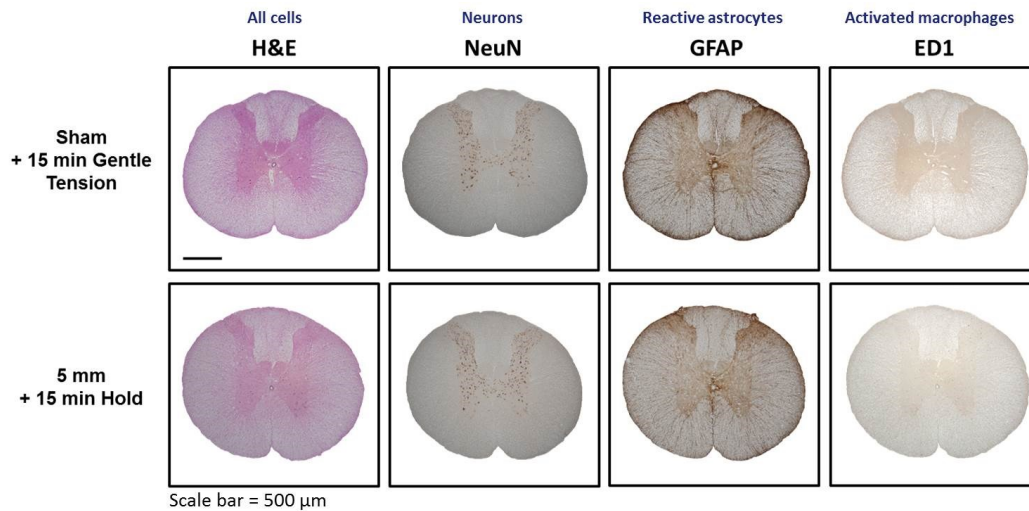
Figure 3.14 Fifteen minute prolonged distraction does not cause spinal cord damage. Transverse spinal cord sections taken from the epicenter of sham and 15 minute prolonged distraction animals and stained for H&E, NeuN, GFAP, and ED1 revealed intact spinal cord tissue and lack of positive staining for reactive astrocytes and activated macrophages. Scale bar = 500 μm.

Quantification of the intensity distribution along a straight line across each H&E-stained spinal cord section revealed that the average signal intensity due to tissue damage in the 7-mm distraction group from the previous study was significantly higher than the average signal intensities in the sham and 15 minute prolonged distraction groups, as shown in Figure 3.15. Conversely, a significant difference between the average signal intensities in the sham and 15

minute prolonged distraction groups was not observed, thereby confirming that a 15 minute prolonged 5-mm distraction does not induce mechanical damage to the spinal cord tissue.
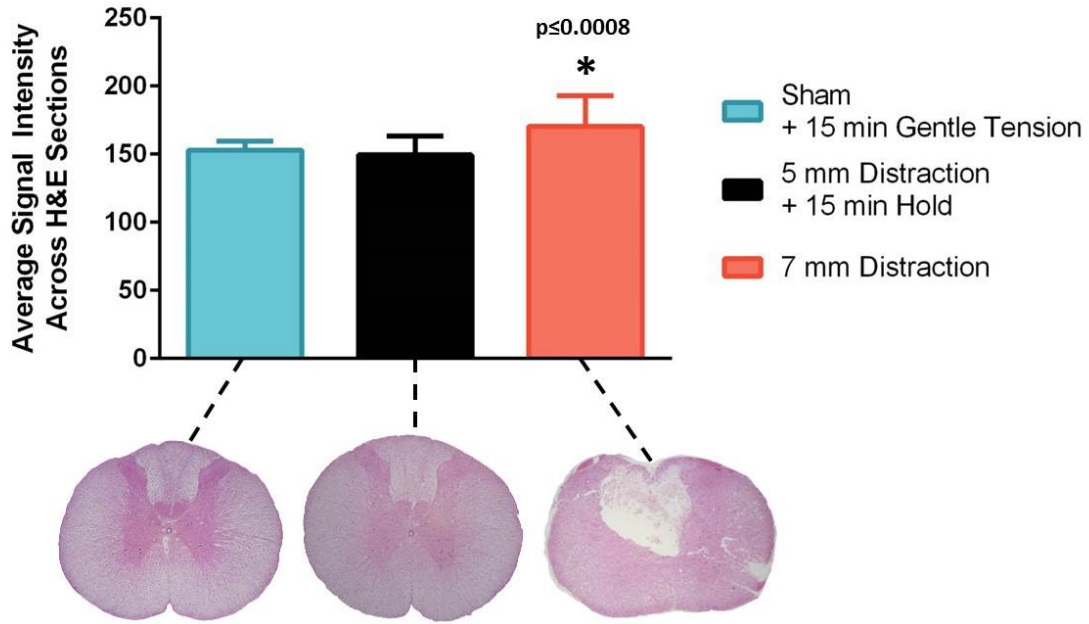
Figure 3.15 Absence of observable tissue loss following 15 minute prolonged distraction. Quantification of average signal intensity across H&E-stained spinal cord sections confirmed a lack of mechanical damage to the spinal cord tissue following a 15 minute prolonged distraction. While tissue damage in animals distracted 7-mm results in significantly higher average signal intensity, the average signal intensities in the sham and prolonged distraction groups are not significantly different. Data represent the average and standard deviation (n = 6 per sham and 15 min prolonged distraction groups). *p≤0.0008 compared to sham/15 minute prolonged distraction.

The lack of tissue loss, neuron-depleted regions, and positive staining for GFAP and ED1 observed at the epicenter was also observed in proximal and distal sections following a 15 minute prolonged distraction, as shown in Figures 3.16, 3.17, 3.18, and 3.19.
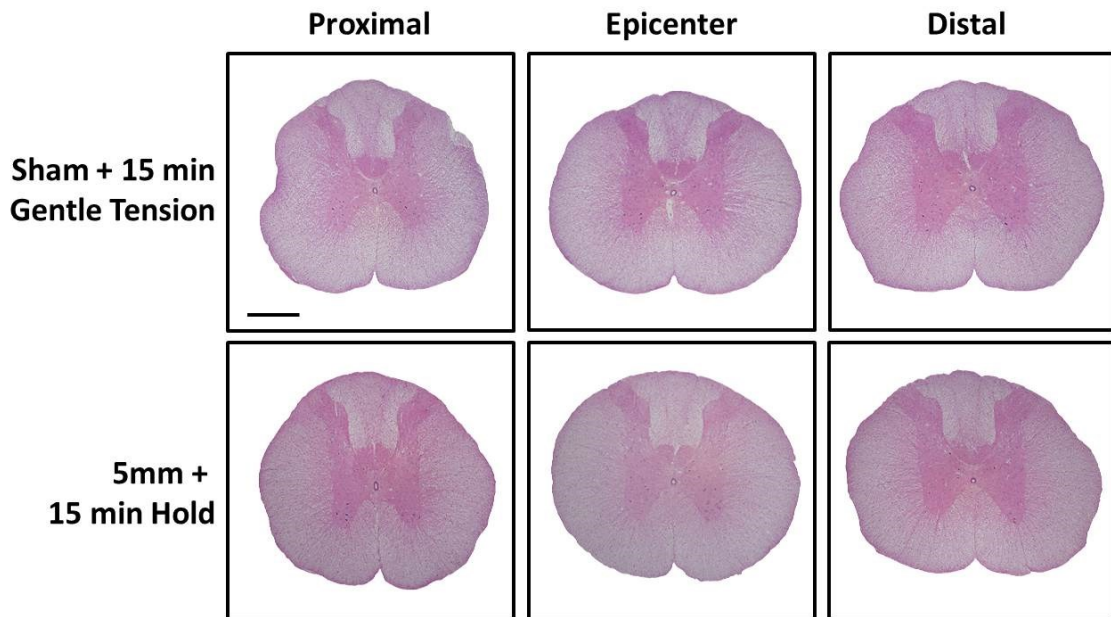
Figure 3.16 H&E-stained spinal cord sections following sham injury and 15 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 15 minute prolonged 5-mm distraction animals stained with H&E reveal intact spinal cord tissue. Scale bar = 500 µm.
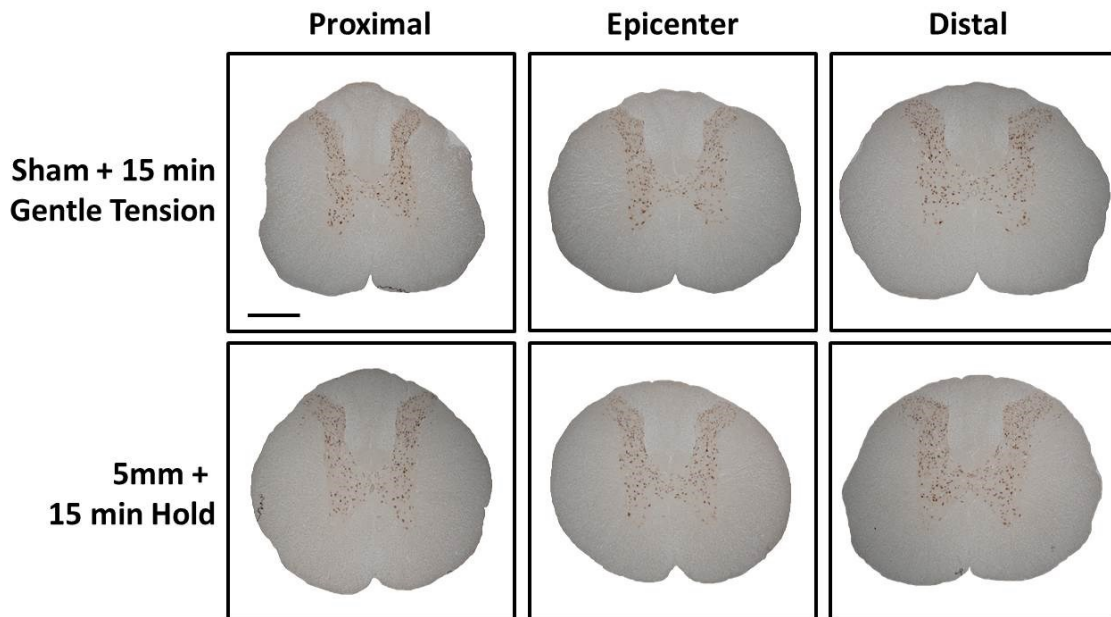
Figure 3.17 NeuN-stained spinal cord sections following sham injury and 15 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 15 minute prolonged 5-mm distraction animals stained with NeuN reveal a lack of neuron-depleted regions. Scale bar = 500 µm.

| | **Proximal** | **Epicenter** | **Distal** |
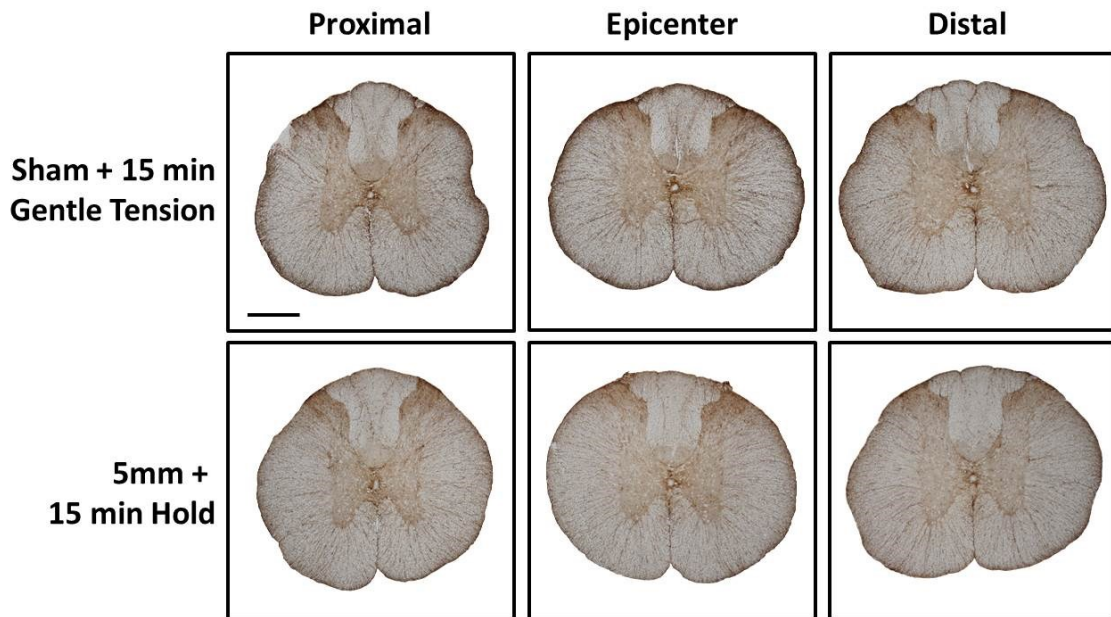
Figure 3.18 GFAP-stained spinal cord sections following sham injury and 15 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 15 minute prolonged 5-mm distraction animals stained with GFAP reveal no increase in reactive astrocytes compared to sham. Scale bar = 500 μm.
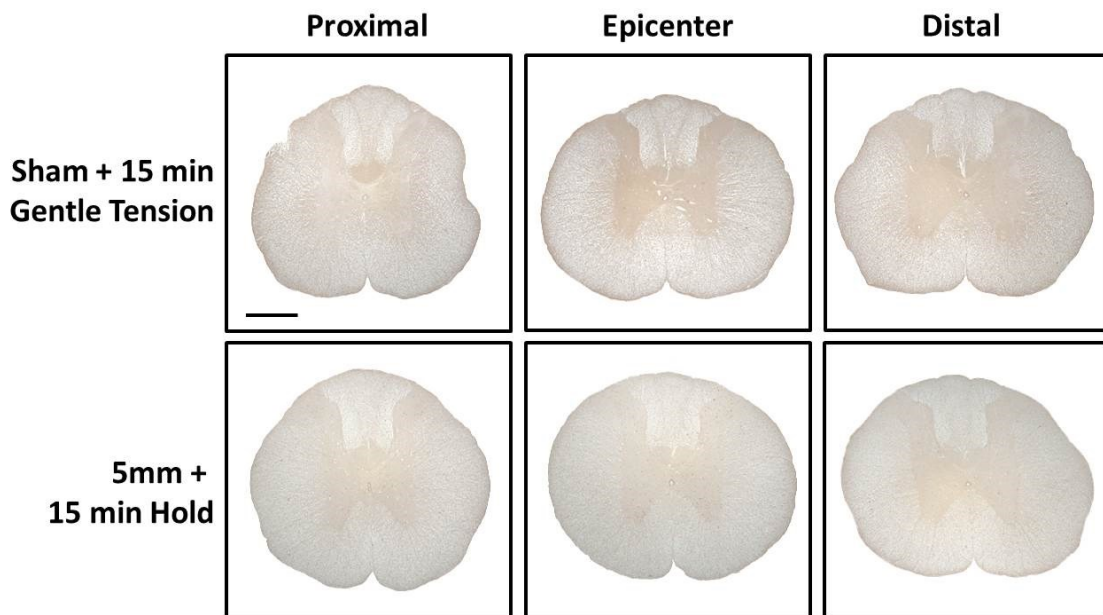
Figure 3.19 ED1-stained spinal cord sections following sham injury and 15 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 15 minute prolonged 5-mm distraction animals stained with ED1 reveal a lack of positive staining for activated macrophages. Scale bar = 500 μm.

3.3.2 Prolonged Distraction Induces Hypoxic Insult

In the absence of observable mechanical tissue damage, we hypothesized that the functional deficits are a result of damage to the vasculature; therefore, we sought to determine the extent to which prolonged distraction SCI induces a hypoxic insult. Representative traces of the distal IP pO_2 levels in response to a sham injury (negative control), an aortic occlusion (positive control), and a 15 minute prolonged distraction are shown in Figure 3.20. No decrease in pO_2 levels was detected in response to a sham injury. Conversely, an immediate, prolonged hypoxic insult was detected in response to an aortic occlusion. As hypothesized, a sharp decline in pO_2 levels within the distal cord parenchyma was detected immediately following the application of distractive force. Interestingly, this sharp decline was transient in nature compared to the prolonged hypoxic insult in response to aortic occlusion.

Figure 3.20 Representative pO_2 recordings distal to the injury site in response to sham injury, aortic occlusion, and 15 minute prolonged distraction. (A) IP pO_2 levels do not decrease following application of gentle tension. (B) Aortic occlusion leads to a prolonged decrease in IP pO_2 levels. (C) The application of distractive force leads to a sharp decline in pO_2 levels that is transient in nature.

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Quantification of the average percent pO_2 decrease 1, 2, 5, 10, 15, and 60 minutes post-injury induction is shown in Table 3.1 and Figure 3.21. The average percent pO_2 decrease one minute post-injury induction was 47.08 ± 11.57 % following the application of distractive force compared to 1.61 ± 1.08 % and 36.26 ± 11.77 % in response to a sham injury and aortic occlusion, respectively. By five minutes post-injury induction, the average percent pO_2 decrease was only 20.51 ± 12.99 % in response to prolonged distraction, and by 60 minutes post-injury induction, the average percent pO_2 decrease in response to prolonged distraction (17.80 ± 18.90 %) was not significantly different from the decrease in response to a sham injury (0.43 ± 0.75 %) or an aortic occlusion (12.58 ± 19.09 %).

Table 3.1 Average Percent pO_2 Decrease Post-Injury Induction

	1 min	2 min	5 min	10 min	15 min	60 min
Sham + 15 min Gentle Tension	1.61 ± 1.08 (4)	0.96 ± 0.97 (4)	0.60 ± 1.04 (4)	1.29 ± 2.24 (4)	1.38 ± 2.39 (4)	0.43 ± 0.75 (4)
15 min Aortic Occlusion	36.26 ± 11.77 (4)*	63.60 ± 12.65 (4)*	91.47 ± 2.37 (4)*	88.81 ± 4.25 (4)*	83.89 ± 5.38 (4)*	12.58 ± 19.09 (4)
5 mm + 15 min Hold	47.08 ± 11.57 (4)*	44.05 ± 19.21 (4)*	20.51 ± 12.99 (4)*+	13.17 ± 11.72 (4)*+	9.17 ± 9.72 (4)+	17.80 ± 18.90 (4)
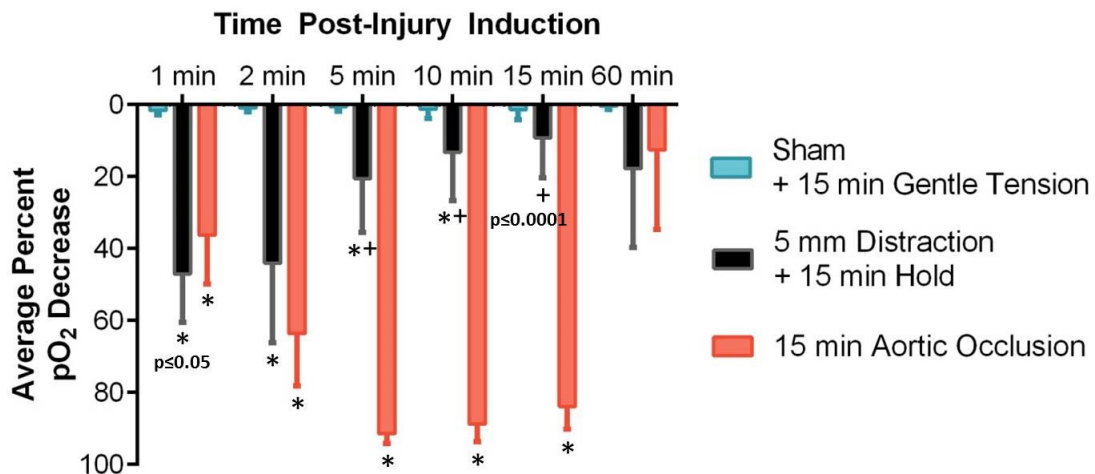
Figure 3.21 Average percent pO_2 decrease 1, 2, 5, 10, 15, and 60 minutes post-injury induction. Data represent the average and standard deviation (n = 4 per group). *p≤0.05 compared to sham and + p≤0.0001 compared to 15 min aortic occlusion.

Interestingly, although the decreased pO_2 levels following the application of distractive force begin to rise within minutes, they do not return to pre-injury levels until clamp removal. Therefore, the difference between the average pre-injury pO_2 levels and the average pO_2 levels following the induction of gentle tension in the sham group was compared to the difference between the average pre-injury pO_2 levels and the average pO_2 levels following the sharp decline but prior to clamp removal in the prolonged distraction group, as shown in Figure 3.22. Quantification revealed that the difference between the average pre-injury pO_2 levels and the average pO_2 levels following the sharp decline but prior to clamp removal in the prolonged distraction group is significantly different than the difference between the average pre-injury pO_2 levels and the average pO_2 levels following the induction of gentle tension in the sham group ($p \leq 0.0150$, one-way ANOVA, data not shown), thereby confirming that a prolonged distraction injury leads to an immediate, transient severe hypoxic insult followed by a mild hypoxic insult for the duration of time held in the distracted position.

The difference between the average pre-injury pO_2 levels and the average post-injury pO_2 levels in the sham group was then compared to the difference between the average pre-injury pO_2 levels and the average post-injury pO_2 levels in the prolonged distraction group, as shown in Figure 3.22. Quantification revealed that the difference between the average pre-injury pO_2 levels and the average post-injury pO_2 levels in the prolonged distraction group is not significantly different than the difference between the average pre-injury pO_2 levels and the average post-injury pO_2 levels in the sham group ($p \leq 0.6559$, one-way ANOVA on the Ranks, data not shown), thereby confirming that the mild hypoxic insult ceases following clamp removal.
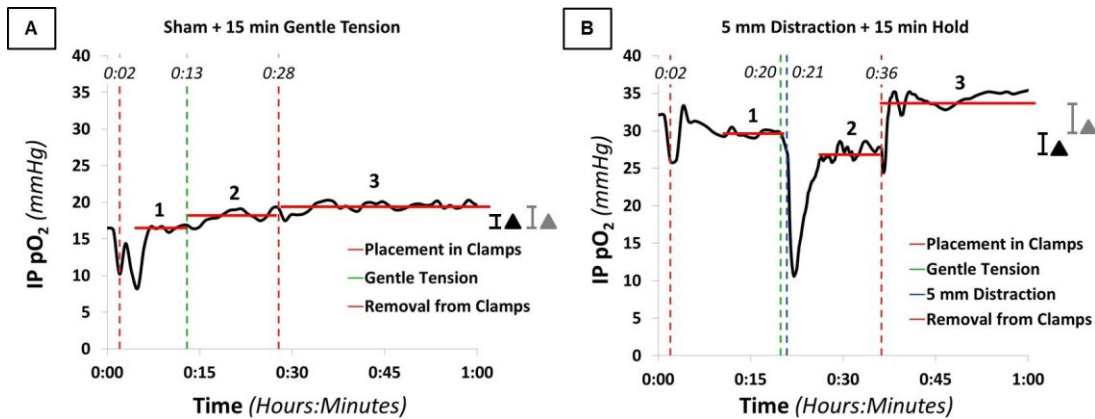
Figure 3.22 Representative pO$_2$ recordings distal to the injury site in response to sham injury and 15 minute prolonged distraction showing regions used for quantification of mild hypoxic insult. Three red lines indicate pre-, during, and post-injury pO$_2$ levels. The difference between the average pre-injury pO$_2$ levels (graph A, red line 1) and the average pO$_2$ levels following the induction of gentle tension (graph A, red line 2) in the sham group, as indicated by the black triangle in graph A, was compared to the difference between the average pre-injury pO$_2$ levels (graph B, red line 1) and the average pO$_2$ levels following the sharp decline but prior to clamp removal (graph B, red line 2) in the prolonged distraction group, as indicated by the black triangle in graph B. In addition, the difference between the average pre-injury pO$_2$ levels (graph A, red line 1) and the average post-injury pO$_2$ levels (graph A, red line 3) in the sham group, as indicated by the gray triangle in graph A, was compared to the difference between the average pre-injury pO$_2$ levels (graph B, red line 1) and the average post-injury pO$_2$ levels (graph B, red line 3) in the prolonged distraction group, as indicated by the gray triangle in graph B.

In animals undergoing sham and prolonged distraction injuries, it was noted that the pO$_2$ levels changed slightly then re-stabilized in response to placement of the animal in the distraction device clamps, a finding that is consistent with a previous study of spinal cord oxygenation following a contusion injury in which pO$_2$ values dropped in response to placement of the animal on the Impactor clamps (Schroeder et al. 2008). Figure 3.23 shows the regions used for quantification of the variability in pO$_2$ levels associated with clamp placement. The variability associated with clamp placement was found to be 6.7 ± 3.21 mm Hg. This variability could possibly result from movement of the probe within the spinal cord as the clamps are placed, so the change in pO$_2$ levels in response to clamp placement was then compared to the change in pO$_2$ levels in response to distraction. Quantification revealed that the change in pO$_2$ levels in response to distraction (15.56 ± 3.84 mm Hg) is significantly greater than the change in pO$_2$ levels in response to clamp placement (p≤0.0011, one-way ANOVA, data not shown),

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thereby confirming that the immediate, transient sharp decline in pO_2 levels observed following

the application of distractive force is not an artifact of probe movement.
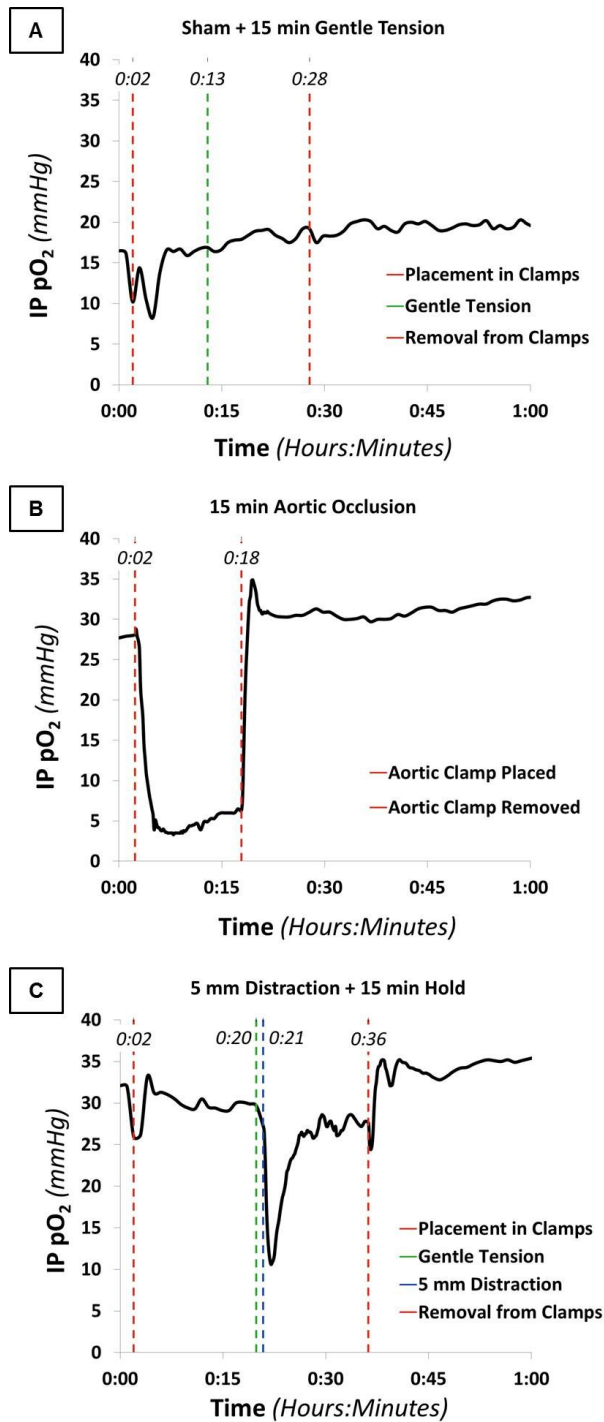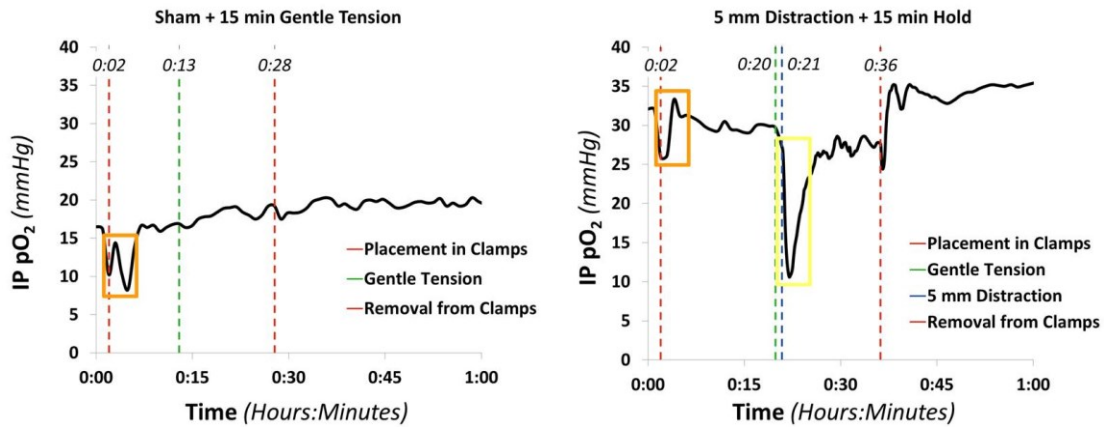

Figure 3.23 Representative pO_2 recordings distal to the injury site in response to sham injury and 15 minute prolonged distraction showing regions used for quantification of variability in pO_2 levels associated with clamp placement. The change in pO_2 levels in response to clamp placement (orange boxes) was compared to the change in pO_2 levels in response to distraction (yellow box).

The individual IP pO_2 values are also shown grouped into three phases (pre, during, and post-injury) for animals undergoing a sham injury, an aortic occlusion, and a prolonged distraction injury, as shown in Figure 3.24. As expected, the immediate, transient severe hypoxic insult following the application of distractive force is less prominent when all IP pO_2 values obtained within the 15 minute period of distraction are grouped together, thereby validating the mild nature of the hypoxic insult following the immediate, transient sharp decline in pO_2 levels.
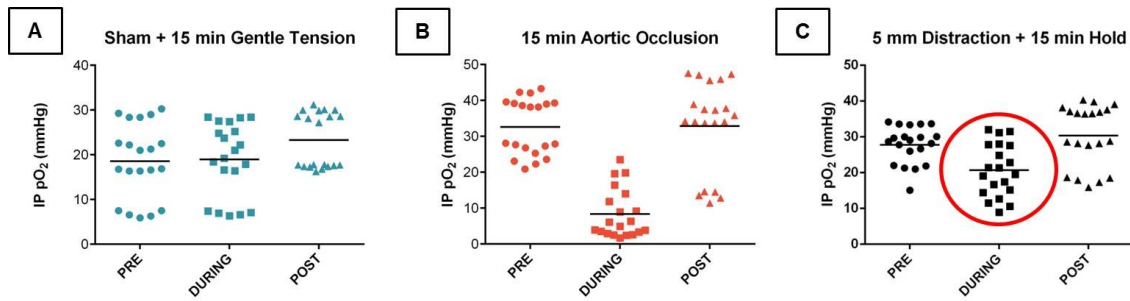
Figure 3.24 Individual IP pO$_2$ values grouped into three phases (pre, during, and post-injury) for animals undergoing sham injury (A), aortic occlusion (B), and prolonged distraction (C). The immediate, transient sharp decline in pO$_2$ levels observed following the application of distractive force is less prominent when all IP pO$_2$ values obtained within the 15 minute period of distraction are grouped together (red circle), thereby validating the mild nature of the hypoxic insult following the immediate, transient sharp decline in pO$_2$ levels.

3.3.3 Prolonged Distraction Leads to Acute Increase in Protein Oxidation

We then aimed to identify events occurring at the intracellular level following a prolonged distraction. Specifically, we examined ROS generation and protein oxidation, two outcomes known to result from secondary injury mechanisms initiated by hypoxia. Interestingly, we did not observe a significant increase in ROS/RNS levels at any of the post-injury time points evaluated (Figure 3.25).
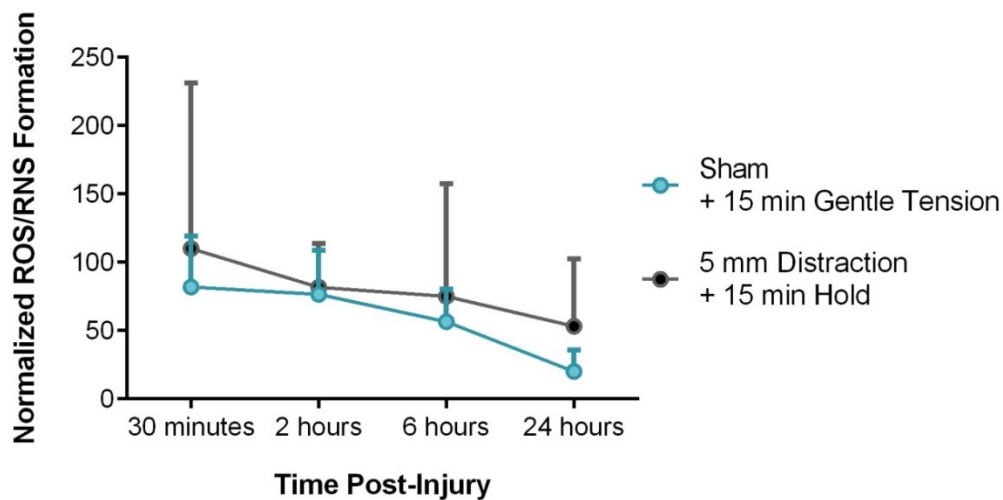
Figure 3.25 Fifteen minute prolonged distraction does not lead to an increase in ROS/RNS levels. Data represent the average and standard deviation (n = 3 and 6 per sham and 15 minute prolonged distraction groups, respectively, per time point).

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However, consistent with a mild hypoxic insult, biochemical analysis revealed an acute increase in protein oxidation following prolonged distraction. Specifically, the average protein carbonyl content in the distraction group was 216% higher than the average protein carbonyl content in the sham group 30 minutes post-injury (Figure 3.26A). A significant difference was not observed at 2, 6, or 24 hours post-injury. The individual percent carbonyl content increases following prolonged distraction over sham injury at each time point are shown to highlight the source of variability at the 6-hour post-injury time point (Figure 3.26B). The actual protein carbonyl content in the sham and prolonged distraction groups and the protein carbonyl content in the prolonged distraction group normalized to the content in the sham group are shown in Figures 3.26B and 3.26C, respectively, for visualization purposes. All statistics were run on the raw data. These data indicate that while the hypoxic insult in response to prolonged distraction is mild, it is sufficient to induce downstream mitochondrial effects.
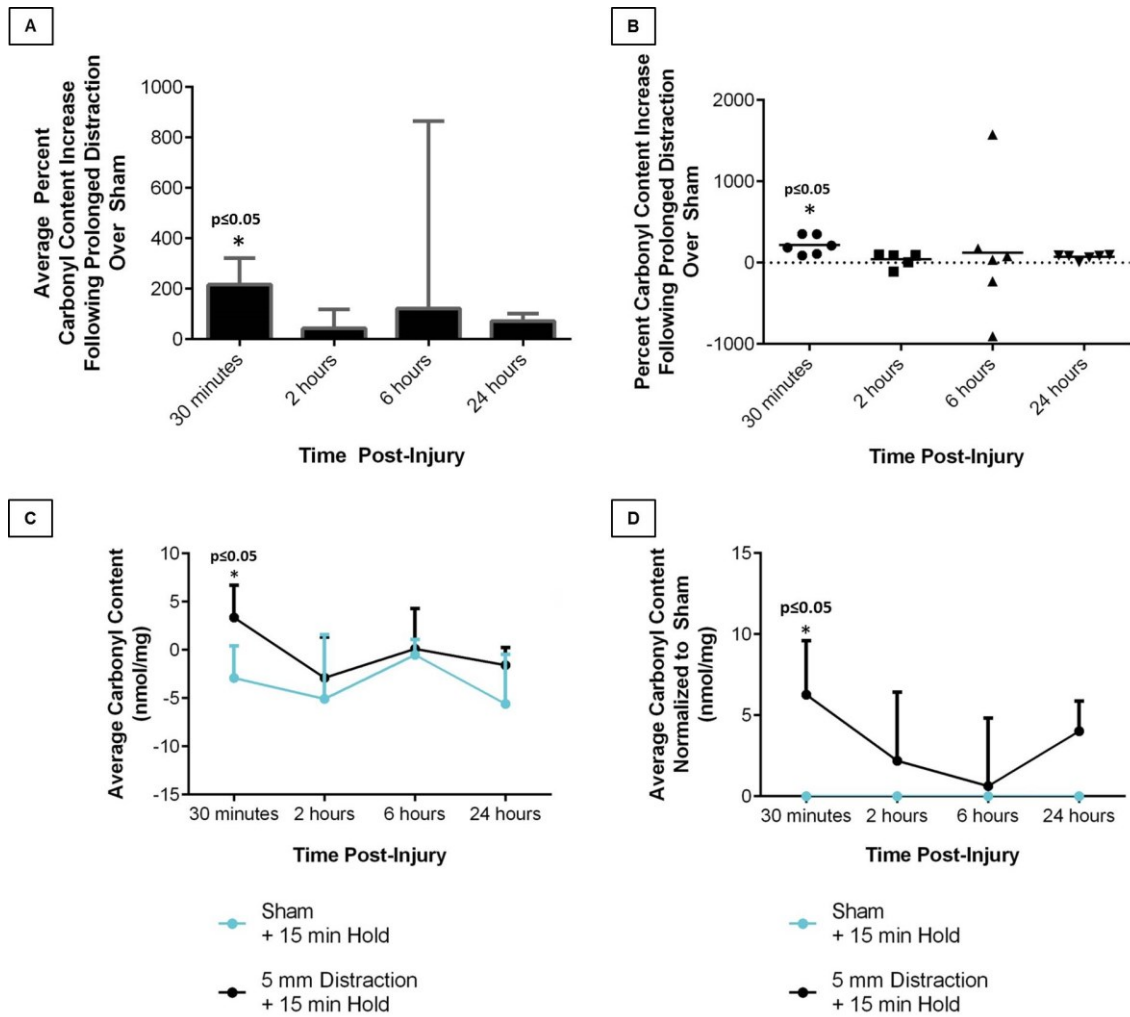
Figure 3.26 Acute increase in protein oxidation following 15 minute prolonged distraction. (A) The average protein carbonyl content is increased by 216% over content in sham group following a 15 minute prolonged distraction 30 minutes post-injury. (B) The individual percent carbonyl content increases following prolonged distraction over sham injury at each time point are shown to highlight source of variability 6 hours post-injury. The actual protein carbonyl content in the sham and prolonged distraction groups (C) and the protein carbonyl content in prolonged distraction group normalized to content in sham group (D) are shown for visualization purposes. All statistics were run on the raw data. Data represent the average and standard deviation (n = 3 and 6 per sham and 15 minute prolonged distraction groups, respectively, per time point). *p≤0.05 compared to sham.

3.4 Discussion

The secondary injury mechanisms activated by the primary injury following SCI are widespread. Currently, there are many neuroprotective strategies being studied to target these varied mechanisms. Among them are sodium channel blockers, including Riluzole, to prevent glutamate excitotoxicity (Wahl et al. 1993, Stutzmann et al. 1996, Schwartz and Fehlings 2002, Wilson and Fehlings 2013). Pharmaceuticals with anti-inflammatory properties, such as minocycline, have also been explored to reduce apoptotic cell death caused by glutamate excitotoxicity and inflammatory mediators (Tikka et al. 2001, Lee et al. 2009, Iwata et al. 2010, Moon et al. 2012). In addition, antioxidants have been studied to mitigate the effects of increased ROS levels (Liu et al. 2009, Emmez et al. 2010, Thaakur and Sravanthi 2010). More recently, metabolic substrates, such as acetyl-l-carnitine, have been evaluated to counter impaired energy metabolism following mitochondrial dysfunction (Zanelli et al. 2005, Patel et al. 2010, Patel et al. 2012). Since the specific injury mechanisms involved in distraction SCI remain largely unknown, tailoring the selection of possible preventative therapeutic interventions has proved to be difficult; therefore, elucidation of those specific mechanisms will allow for better targeting of future neuroprotective strategies.

The realization of the prevalence of neurologic deficit following scoliosis corrective surgery emphasized the need to determine the underlying cause, and in 1971, it was postulated that the two likely contributions to neurologic deficit following the surgical correction of scoliosis are mechanical damage to the spinal cord tissue and/or mechanical damage to the vasculature of the spinal cord (Keim and Hilal 1971). Here we report the use of a more clinically relevant animal model of distraction SCI in evaluating the independent contributions of tissue damage and vascular compromise for the purpose of elucidating the specific injury mechanisms. The results of this study are substantial in light of our previous study in which the acute injury response to a graded distraction injury was characterized. Previously, a 5-mm distraction resulted in a significant reduction of TcMEP amplitude and a highly variable functional deficit

because dislocation, rather than a purely linear distraction, occurred in several of the animals. Modifications to the clamping mechanism of the UTA Spine Distractor and reduction of the distraction speed led to a more controlled, reproducible injury. The prolonged (15 minute) distraction aimed to more closely mimic surgical conditions in which tension is applied to the spinal column throughout much of the procedure. These changes to the injury paradigm resulted in linear distraction with no dislocation upon release of the tension. This method induced a much less severe injury than previously reported as evidenced by sustained electrocompetency of the spinal cord, no gross tissue loss and mild changes to locomotor function. Thus, this model is more capable of isolating vascular compromise from tissue damage.

Previous studies on distraction SCI have focused on determining the effect of distraction on SCBF for the purpose of evaluating the extent of vascular compromise. Specifically, it has been shown that distraction, when associated with a noticeable change in somatosensory and/or motor-evoked potentials, leads to a reduction in blood flow (Dolan et al. 1980, Kling et al. 1985, Kling et al. 1986, Naito et al. 1992); however, as mentioned earlier, these studies were limited by inadequate models and insufficient characterization of the injury. To improve upon these studies and further the understanding of the extent of vascular compromise, we evaluated the change in spinal cord oxygenation in our model through direct measurement of the distal IP pO_2 levels in response to prolonged distraction. The range of pre-injury pO_2 levels prior to application of gentle tension or insertion of the aortic clamp were consistent with previously reported pre-injury levels obtained using the same oxygen electrode in the thoracic region of a rat spinal cord (Schroeder et al. 2008). As expected, the electrode did not detect a reduction in pO_2 levels in response to a sham injury but did record an immediate, continuous hypoxic insult in response to an aortic occlusion, thereby validating the hypoxic insult detected in response to a prolonged distraction. Since it is well established that the mechanism of autoregulation in the spinal cord maintains constant blood flow through the cord

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through vasoconstriction and dilation during periodic changes in systemic blood pressure or CO_2 concentrations (Smith et al. 1969, Kindt 1971, Kobrine et al. 1975, Martirosyan et al. 2011), it is likely that autoregulation is a contributing factor to the mild nature of the hypoxic insult following the immediate, transient sharp decline in pO_2 levels observed in response to a prolonged distraction.

Although it remains uncertain how distraction causes ischemic damage to the spinal cord, there is evidence to suggest that mechanical tissue damage may be distinguishable from mechanical damage to the vasculature based on the time it takes for motor-evoked potentials to become abnormal following injury (Owen et al. 1990, Naito et al. 1992, Kai et al. 1993). Specifically, Owen et al. (1990) characterized a slower effect of distraction on SCBF as predominantly an insult to the vasculature in which loss of sensory and motor-evoked potentials were not observed until 20 minutes post-injury; conversely, a faster effect of distraction on SCBF was characterized as predominantly an insult to the tissue with loss of potentials occurring within 4 minutes post-injury, coupled with observation of structural damage to the cord. In this study, the mild nature of the hypoxic insult in response to a prolonged distraction may explain why changes in TcMEPs were not observed immediately following injury. Regardless, the fact that a mild functional deficit resulted from this mild hypoxic insult suggests the initiation of specific secondary injury mechanisms.

Since it has been established that an ischemic insult can trigger mitochondrial dysfunction and oxidative stress mechanisms (Fiskum 2000, Pandya et al. 2011), we evaluated this possibility as an activated secondary signaling mechanism following distraction in light of the absence of reactive gliosis and activated macrophages. Mitochondria regulate a multitude of pathways including cell maintenance, survival, and energy production, as well as apoptotic pathways (Fiskum 2000, Nicholls and Budd 2000, Sullivan et al. 2005). Mitochondrial dysfunction following SCI or other damage can lead to a depletion of energy (ATP) production, as well as an increase in ROS levels (Turrens 2003, Brookes et al. 2004, Sullivan et al. 2005,

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Mcewen et al. 2011). As mitochondrial complex respiration fails, steady-state levels of free radicals can increase and, in turn, promote DNA, protein and lipid oxidation, thereby increasing cellular damage (Richter et al. 1988, Rubbo et al. 1994, Ledoux et al. 1999, Stadtman and Levine 2000). Protein oxidation products, protein carbonyls, are among the most common type of damage used to infer oxidative stress and mitochondrial dysfunction (Shacter 2000, Stadtman and Levine 2000). Therefore, we evaluated changes in ROS levels and protein carbonyl content as an indirect method to assess mitochondrial function following prolonged distraction.

In this preliminary study, we did not observe an increase in ROS levels following prolonged distraction; however, we did observe an acute increase in protein carbonyl content at 30 minutes post-injury. In interpreting the negative results of the ROS assay, it is important to consider the following. Despite the fact that an increase in ROS in spinal cord tissue has been observed following compression SCI (Xu et al. 2005), prolonged distraction is a very different injury paradigm so a strong correlation to previous studies may not be valid in this study. However, the lack of increase in ROS observed in this study is surprising given the fact an acute increase in protein oxidation was observed. One possible explanation for this result relates to the short half-life of ROS. It is known that ROS are transient and that most free radicals are stable for only seconds (Devasagayam et al. 2004). In fact, the OxiSelect *In Vitro* ROS/RNS Assay Kit used to assess ROS formation in this study mainly detects hydrogen peroxide (H_2O_2) in samples because it is one of the more stable free radicals (D'autreaux and Toledano 2007). While the assay kit can also detect peroxyl radical ($RO_2\cdot$), nitric oxide ($NO\cdot$), and peroxynitrite anion ($ONOO^-$), detection of free radicals with shorter half-lives may have been impaired if the free radicals were not stable during the tissue preparation, thus resulting in a low signal. In addition, if prolonged distraction does induce a very small yet significant increase in H_2O_2, for example, it would be difficult to distinguish this increase as significant because the relative fluorescent units (RFU) for varying low concentrations of H_2O_2 are very

similar and, thus, difficult to differentiate. In short, additional studies are warranted to further evaluate free radical formation to confirm or deny the result obtained in this study.

The acute increase in protein carbonyl content observed in this study following prolonged distraction over that of the sham control is similar to a previous observation showing an early increase in protein oxidation in rat spinal cord tissue one hour following a contusion injury (Aksenova et al. 2002). While further studies are warranted to examine the extent of oxidative stress and, specifically, mitochondrial function following prolonged distraction, the results of this study have led to the proposed injury schematic shown in Figure 3.27 in which distraction SCI induces hypoxia, which results in acute protein oxidation.
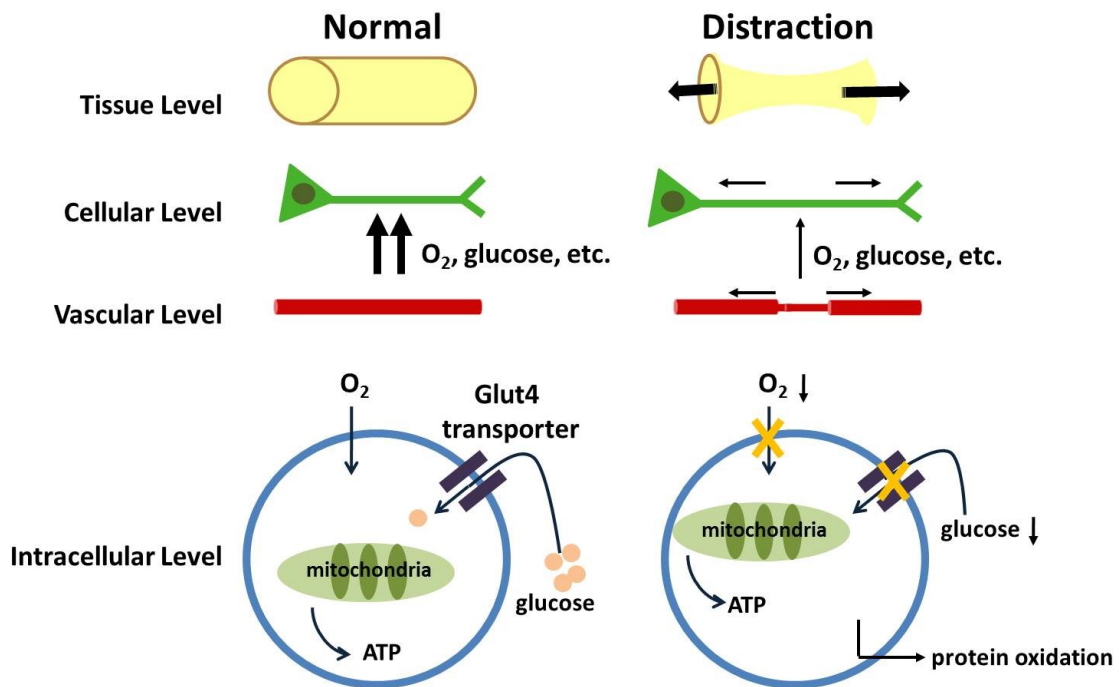
Figure 3.27 Proposed injury schematic of distraction SCI. Upon injury, blood vessels are partially occluded, leading to hypoxia, which in turn results in downstream protein oxidation.

3.5 Conclusion

In summary, the secondary injury mechanisms following SCI can initiate a number of converging and diverging molecular and biochemical cascades, making any attempt to target neuroprotective therapeutics to one pathway extremely difficult. The results of this study have verified the ability of this model to isolate mechanical damage to the vasculature from mechanical damage to the tissue and rendered a more comprehensive understanding of the injury mechanisms involved in distraction SCI. This vital knowledge will better enable the selection of highly target-specific treatment strategies aimed at minimizing and/or preventing injury to the spinal cord during spine deformity surgery.

CHAPTER 4

CONCLUSION

4.1 Summary

The multidirectional stress forces imparted to the spinal cord, including those of distraction, during scoliosis corrective surgery may result in the development of new neurological deficits. IONM is used to alert the surgeon to possible complications; however, the complex nature of SCI involves a primary mechanical insult to the tissue and/or vasculature that may go undetected by monitoring. This primary injury is followed by the activation of widespread secondary injury mechanisms. The majority of animals models being used to study the pathophysiology of SCI are rat models of transection or contusion injury, and the few published models of distraction injury are limited by high variability and little to no acute characterization. As a result, the specific injury mechanisms involved in distraction SCI remain largely unknown, increasing the difficulty of tailoring the selection of possible preventative therapeutics. Thus, in the context of scoliosis corrective surgery, the establishment and characterization of an animal model capable of reproducibly mimicking the neurological deficits that can result from the application of distractive forces to the spinal cord during scoliosis correction serves two purposes: 1) to gain a better understanding of the specific injury mechanisms involved in the onset of distraction-induced SCI and 2) to determine the efficacy of various treatment strategies designed to minimize and/or prevent the onset of deficits following distraction-induced injury to the spinal cord.

While the establishment of the animal model presented in this dissertation is focused on achieving a reliable model of distraction SCI that can be used to characterize the under-studied paradigm of distraction SCI and ultimately be used for the testing of neuroprotective strategies, it is worth comparing the clinical parameters with this animal model, as shown in Table 4.1.

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Information regarding the typical length of correction in spine deformity surgery and the nature of the resulting functional deficits observed was obtained through personal communication with Daniel J. Sucato, M.D., M.S. at TSRHC in Dallas, TX. Information regarding the total surgery time and the total number of TcMEP stimulations per surgery used in the clinic at Texas Scottish Rite Hospital for Children (TSRHC) was obtained through personal communication with Elizabeth Van Allen M.S., R.EPT., CNIM at TSRHC in Dallas, TX. Finally, information regarding the intraoperative MAP used in the clinic at TSRHC was obtained through personal communication with Cynthia Woerz, M.D. at TSRHC in Dallas, TX.

Table 4.1 Comparison of Clinical Parameters with Animal Model

Parameter	Clinic	Animal Model	Percentage of Approximation
Average Segment Length of Correction	10% length of correction in thoracic region	5-mm distraction at T10 = 10% thoracic segment	100%
Average Total Surgery Time	4-8 hours (AIS), 10-16 hours (severe deformity)	1.5 hours	15%
Average Total No. TcMEP Stimulations Per Surgery	40 stimulations	20 stimulations	50%
Average Intraoperative Mean Arterial Pressure During Correction	80 mm Hg	N/A	N/A
Resulting Functional Deficits	Predominantly mild	Consistently mild	100%

The first parameter for comparison is the segment length of correction. In the clinic, the average segment length of correction is approximately 10% in the thoracic region (personal communication with Daniel J. Sucato, M.D., M.S., Texas Scottish Rite Hospital for Children). In the rat spinal column, 5-mm is approximately 10% of the thoracic segment; therefore, the 5-mm distraction paradigm utilized in this animal model correlates well with the typical length of correction in pediatric patients undergoing scoliosis corrective surgery.

The second parameter for comparison is the total surgery time. The total surgery time in the clinic, which includes exposure, removal of facet joints, placement of instrumentation, correction of the curve, and placement of pieces of bone graft for the fusion process, varies depending on the severity of the case. In cases of AIS, the total surgery time typically ranges

from 4 to 8 hours, depending on the surgeon. In cases of more severe deformity in which more aggressive procedures, such as VCR, are implemented, the total surgery time can be as long as 16 hours. Conversely, in this animal model, the total surgery time is typically 1.5 hours from the time the animal is anesthetized to the completion of the procedure.

The third parameter for comparison is the total number of TcMEP stimulations during surgery. In the clinic, patients typically require two or three stimulations with an average train of six pulses per second on each side to achieve maximal responses. The frequency with which TcMEPs are checked throughout a given surgery depends on the surgeon and the severity of the case. Some surgeons prefer to check TcMEPs repeatedly, as in every few minutes, especially during the placement of screws and correction phases. Other surgeons check TcMEPs at the conclusion of each major step of the surgery (i.e., when all screws have been placed, after each rod has been placed, after correction, and during closing.) Thus, the total number of TcMEP stimulations during a spine deformity surgery is typically around 40-45 stimulations. In this animal model, TcMEPs are checked at least twice on each side with a train of three stimulation pulses at the following time points: prior to the start of surgery, upon exposure of the vertebral bodies prior to placement in the clamps, after placement in the clamps and gentle tension prior to distraction to ensure the spinal cord is intact pre-injury, following removal from the clamps, and then after closure. Thus, the total number of TcMEP stimulations during a surgery is, on average, around 20 stimulations.

The fourth parameter for comparison is the MAP of the patient during the corrective phase of the surgery. In the clinic, hypertension is often induced during correction, despite increased blood loss, to prevent ischemic damage to the spinal cord. Specifically, while it depends on their normal pre-surgical blood pressure, a patient's MAP is typically maintained at ≥ 60 mm Hg throughout the surgery but is raised to ≥ 80 mm Hg during correction. In this animal model, controlled hypertension is not induced during the period of distraction.

The fifth parameter for comparison is the nature of the resulting functional deficits. In the clinic, the majority of neurological deficits observed following scoliosis corrective surgery are typically mild in nature (personal communication with Daniel J. Sucato, M.D., M.S. at TSRHC in Dallas, TX). Likewise, in this animal model, the functional deficit observed following a prolonged distraction injury is consistently mild in nature.

Because exact duplication of the surgical parameters implemented during scoliosis corrective surgery is not feasible in a non-scoliotic rat and is not the goal of this research project, the animal model presented in this dissertation provides an excellent framework around which to investigate the under-studied paradigm of distraction, which is known to occur during the correction of scoliotic spines. As such, three specific hypotheses were tested in these studies. The first hypothesis that a bidirectional distraction SCI would mimic the neurological deficits that can result from the application of distractive forces during spine deformity surgery was tested through characterization of the acute injury response to a graded distraction injury. Following 3, 5, and 7-mm distractions, we observed a graded acute injury response in which TcMEP amplitude reduction, functional deficit, and percent tissue loss were directly proportional to the distance of distraction. Despite the fact that further modifications were necessary to better mimic the application of distractive forces to the spine utilized during spine deformity surgery, the results of this study demonstrate that the UTA Spine Distractor can be used to apply highly controlled bidirectional distraction forces to the rat spine. Modifications to the distraction device and injury paradigm resulted in a model with the ability to isolate mechanical damage to the vasculature from mechanical damage to the spinal cord tissue. The second hypothesis that a prolonged spinal distraction would introduce a hypoxic insult to the spinal cord was then tested through direct measurement of the oxygenation levels in the distal cord parenchyma. We recorded a transient sharp decline in pO_2 levels in the distal cord parenchyma immediately following the application of distractive force. This sharp decline was followed by a mild hypoxic insult for the duration of the time held in the distracted position. The third hypothesis that the

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mechanical disruption and period of low oxygenation would be sufficient to negatively affect mitochondrial function, leading to increased levels of ROS and an increase in protein oxidation, was tested through biochemical analysis. Though we did not observe an increase in ROS formation, we did observe an acute increase in protein carbonyl content, a known indicator of oxidative stress.

Results from these studies have rendered a more comprehensive understanding of the specific injury mechanisms initiated following distraction. This vital knowledge, in combination with this highly reproducible model, will greatly improve the future development of highly target-specific neuroprotective strategies aimed at reducing and/or preventing unintended neurological damage to the spinal cord during scoliosis correction.

<center>4.2 Future Directions</center>

In light of the results presented in this dissertation, the future directions of this research project are two-fold. The first objective entails further modification of the injury paradigm to increase the deficit for the purpose of creating a larger separation between injured and non-injured animals so that any improvement elicited by a neuroprotective agent can be better appreciated. The second objective entails utilizing the animal model to test potentially neuroprotective strategies aimed at minimizing and/or preventing the onset of neurological deficits following spine deformity correction.

4.2.1 Increasing Functional Deficit

With the current injury paradigm utilized in this animal model, we observe a consistent, reproducible mild functional deficit following prolonged distraction. While the majority of neurological deficits observed in the clinic following scoliosis corrective surgery are typically mild in nature (personal communication with Daniel J. Sucato, M.D., M.S. at TSRHC in Dallas, TX), a greater deficit will be needed in order to better appreciate the difference between injured and protected animals once the testing of neuroprotective strategies begins. A few proposed

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modifications to the injury paradigm that may contribute to an increased functional deficit are described below.

The first proposed modification is aimed at achieving a more severe yet pure distraction injury. This would involve stabilizing the column such that a longer distraction distance can be induced without causing a dislocation injury. For example, following the first study in which a graded distraction injury was characterized, it was noted that a 7-mm distraction resulted in dislocation in all animals; this dislocation injury was likely the cause of the massive mechanical damage to the spinal cord tissue observed following histological analysis. Therefore, it is likely that a 7-mm distraction injury, in the absence of dislocation, may result in increased functional deficits without compromising the structural integrity of the cord.

The second proposed modification is aimed at exacerbating the injury through an increased amount of TcMEP stimulation during the surgical procedure. Many studies on bioenergetics have established that neuronal activity is a largely ATP-consuming process (Attwell and Laughlin 2001, Foo et al. 2012). The forced neuronal firing associated with IONM stimulation accelerates energy store depletion. Thus, it is possible that overstimulation of the cord through an increased stimulation voltage and/or an increased number of stimulations will exacerbate the effects of the hypoxic insult following prolonged distraction. In evaluating the plausibility of this proposed modification, it is important to consider that the incidence of neurologic deficits in the clinic is relatively low despite the use of IONM. In fact, the incidence of neurologic deficits has actually decreased as a result of IONM implementation in the clinic. That being said, overstimulation of the cord may or may not contribute to an increased deficit following prolonged distraction.

The third proposed modification involves the induction of hypotension during the surgical procedure. The use of controlled hypotension was a standard clinical procedure to minimize blood loss during scoliosis corrective surgery for many years (Anderson 1955, Mcneill et al. 1974, Anderson 1978). However, there is concern that induced hypotension lowers SCBF,

posing greater risk of ischemic injury to the spinal cord (Ewards and Flemming 1975, Lindop 1975), so previous studies have evaluated the effect of distraction with and without hypotension on SCBF. Kling et al. (1985) investigated the effects of sodium nitroprusside and halothane-induced systemic hypotension on SCBF with and without distraction SCI in dogs. It was discovered that SCBF was decreased significantly following a 50% reduction in MAP but returned to a normal value within 35 minutes, which is suggestive of autoregulation in the spinal cord (Kling et al. 1985). It was then observed that a distraction of 1-2 cm, correlating to clinical distraction during Harrington instrumentation, did not significantly reduce SCBF when there was a minimum of 45 minutes between induction of hypotension and distraction (i.e., following the induction of autoregulation) (Kling et al. 1985). A subsequent study revealed that trimethaphan (Arfonad)-induced hypotension resulted in a more permanent decrease in SCBF, suggestive of impaired autoregulation (Kling et al. 1986). These findings suggest that the induction of hypotension to 50% of the normal MAP immediately before prolonged distraction may compromise the surrounding vasculature and exacerbate the ischemic injury.

The proposed modifications to the injury paradigm described above are aimed at contributing to an increased functional deficit following prolonged distraction. A greater functional deficit will enhance the contrast between injured and non-injured animals so that any improvement elicited by a neuroprotective agent can be better appreciated.

4.2.2 Testing Neuroprotective Strategies

In addition to reproducibly mimicking the neurological deficits that can result from the application of distractive forces to the spinal cord during scoliosis correction for the purpose of elucidating the specific injury mechanisms, the establishment of this animal model also serves to provide a platform for determining the efficacy of various therapeutic strategies aimed at minimizing and/or preventing the effects of distraction SCI. Currently, there are two groups of therapeutic strategies being studied to enhance functional recovery following SCI: strategies designed to increase axonal regeneration across a spinal cord lesion and strategies designed to

protect the cord from the deleterious effects of secondary injury mechanisms (Fehlings et al. 2012). Since distraction SCI primarily leads to vascular compromise in the absence of tissue disruption, the therapeutic strategies tested in this animal model will be strategies aimed at targeting secondary injury mechanisms. There are many neuroprotective strategies being studied to target the widespread secondary injury mechanisms known to occur following SCI. The following sub-sections will highlight a few of these strategies.

4.2.2.1 Sodium Channel Blockers

Voltage-sensitive sodium channel blockers have increasingly been studied to mitigate the effects of the disruption of sodium homeostasis and glutamate excitotoxicity following SCI (Gangemi et al. 2000, Schwartz and Fehlings 2002). One potent sodium channel blocker, tetrodotoxin (TTX), has been associated with white matter sparing and improved hind limb functional recovery following a contusion injury to the rat spinal cord (Teng and Wrathall 1997, Rosenberg et al. 1999); however, it is not an ideal candidate for clinical testing due to its neurotoxic properties. A more notable sodium channel blocker, Riluzole, which is FDA approved for the treatment of amyotrophic lateral sclerosis (ALS), has been shown to improve functional recovery and decrease tissue loss to a greater extent than other sodium channel blockers, such as phenytoin, when administered after a compression injury to the rat spinal cord (Stutzmann et al. 1996, Schwartz and Fehlings 2001). Because Riluzole is already FDA approved and has shown a greater therapeutic promise in rat models of SCI, a two-year phase I clinical trial was performed to characterize the pharmacokinetics of Riluzole administration in adults with acute SCI (Chow et al. 2012, Fehlings et al. 2012, Wilson and Fehlings 2013). Following administration of 50 mg Riluzole either orally or enterally every 12 hours for two weeks, a higher clearance and larger volume of distribution of the drug was observed in SCI patients compared to patients with ALS (Chow et al. 2012). The neurological outcomes of the patients in this trial are expected to be published this year. In summary, Riluzole has emerged as one of the most promising neuroprotective drugs currently under investigation (Cheah et al. 2010).

4.2.2.2 Anti-inflammatories

Anti-inflammatory agents have also been extensively studied to reduce apoptotic cell death caused by glutamate excitotoxicity and inflammatory mediators (Iwata et al. 2010, Moon et al. 2012). Minocycline, for example, has been shown to decrease pro-inflammatory cytokines and enhance motor function following contusion injury in a rat (Lee et al. 2003, Teng et al. 2004). Another anti-inflammatory agent studied heavily over the past several years is methylprednisolone (MP), a steroid that has been shown to inhibit the early infiltration of immune cells following SCI (Bartholdi and Schwab 1995). Interestingly, MP has also been shown to inhibit lipid peroxidation, as well as increase functional recovery and tissue sparing following SCI (Means et al. 1981, Braughler and Hall 1982). There have been at least three clinical trials investigating the effects MP in the treatment of acute SCI (Bracken et al. 1984, Bracken et al. 1985, Bracken et al. 1992, Bracken et al. 1997). Several aspects of these trials, however, have been called into question (Nesathurai 1998, Coleman et al. 2000, Short et al. 2000, Sayer et al. 2006), so the efficacy of MP in treating human SCI is still up for debate. In fact, the use of anti-inflammatories in treating SCI altogether remains controversial because of the debate over the role of the inflammatory response following SCI.

4.2.2.3 Antioxidants

Antioxidants have been investigated to mitigate the effects of increased ROS/RNS levels and oxidative stress mechanisms (Linseman 2009, Jia et al. 2012). Endogenous biological compounds, such as vitamins C and E, are some of the main antioxidants currently being studied. Alpha-tocopherol (vitamin E) has been reported to promote improved motor function and increased SCBF following a compression injury in the rat spinal cord (Iwasa et al. 1989, Al Jadid et al. 2009). In addition, vitamin E has also been shown to minimize oxidative stress markers, including malondialdehyde (MDA), and improve functional recovery following an ischemia/reperfusion injury in a rat (Morsy et al. 2010). While systemic administration of endogenous antioxidants have shown therapeutic promise, one emerging trend in antioxidant

therapy is the modification of these endogenous antioxidants for the selective targeting of specific ROS-producing intracellular organelles, such as mitochondria (Smith et al. 1999, Kelso et al. 2001, Linseman 2009).

4.2.2.4 Metabolic Substrates

More recently, metabolic substrates have been evaluated to counter impaired energy metabolism and subsequent reduction in energy substrates resulting from ischemia-induced mitochondrial dysfunction and oxidative stress. Among them are substrates that promote aerobic energy metabolism, such as acetyl-l-carnitine which has been shown to stabilize mitochondrial function, resulting in increased tissue sparing, when administered intraperitoneally following contusion injury in a rat (Zanelli et al. 2005, Patel et al. 2010, Patel et al. 2012). Another well-studied substrate, creatine, has also been shown to boost mitochondrial function and decrease the amount of scar tissue when administered orally prior to a contusion injury in a rat (Hausmann et al. 2002, Rabchevsky et al. 2003). Thus, while the majority of neuroprotective therapies being studied in the context of SCI target the downstream by-products of secondary injury mechanisms, there is growing evidence to suggest that targeting the dysfunction of organelles, such as mitochondria, upstream of the secondary injury by-products may provide equal if not more therapeutic benefit (Rabchevsky et al. 2011).

4.2.2.5 The Future of Neuroprotective Strategies

The widespread and overlapping nature of the cellular and molecular changes following SCI increases the difficulty in achieving the desired functional outcomes through the targeting of one specific secondary injury pathway. Thus, future neuroprotective strategies will likely need to be combinatorial in order to successfully improve functional outcomes following acute SCI.

4.3 Overall Conclusions

In addition to providing valuable insight into the specific injury mechanisms involved in distraction SCI for the purpose of enabling the selection of highly target-specific therapeutics, the animal model presented in this dissertation has also provided a reliable platform on which to

test those neuroprotective strategies. Further modifications to the model will be aimed at increasing the functional deficit following prolonged distraction to allow for the reliable testing of possible therapeutic interventions designed to prevent neurological deficits following spine deformity surgery.

APPENDIX A

PRELIMINARY EVALUATION OF THE EFFECT OF TIME IN DISTRACTED POSITION
ON INJURY SEVERITY

Following a 15 minute prolonged 5-mm distraction, we observed a consistent, mild functional deficit. While this deficit is highly reproducible, a greater deficit will be needed in order to better appreciate the difference between injured and protected animals once the testing of neuroprotective strategies begins. In the clinic, spine deformity patients are commonly held in an extended period of traction to aid in the alignment of the spine; therefore, we hypothesized that a longer hold in the distracted position would exacerbate injury severity in this animal model. To test this hypothesis, we evaluated the acute injury effects of a 30 min prolonged 5-mm distraction injury.

All methods were performed as previously described in Chapter 3. Briefly, three female Long-Evans rats (275-325 g) underwent a 30 minute prolonged 5-mm distraction injury alongside the animals that underwent sham and 15 minute prolonged distraction injuries in the previous study. The same three outcome measures were used to evaluate the acute injury effects of a 30 minute prolonged distraction, including electrophysiological monitoring, behavioral analysis, and histological analysis. All results obtained for 30 minute prolonged distraction animals were compared to the results obtained for sham animals described in the previous study. The extent of hypoxic insult following a 30 min prolonged distraction injury was also evaluated in one animal as a preliminary step to assess the effect of time held in a distracted position on the severity of the vascular compromise.

Similar to the results obtained following a 15 minute prolonged distraction, electrophysiological monitoring revealed that TcMEP amplitudes recorded both proximally (data not shown) and distally to the injury site are not significantly affected by a 30 minute prolonged distraction injury, as seen by the representative traces shown in Figure 1A. While there appears to be a downward trend, quantification confirmed that the average percent TcMEP amplitude decreases recorded distally to the injury site following a 30 minute prolonged distraction injury are not significantly different from those recorded distally following a sham injury (Figure 1B).
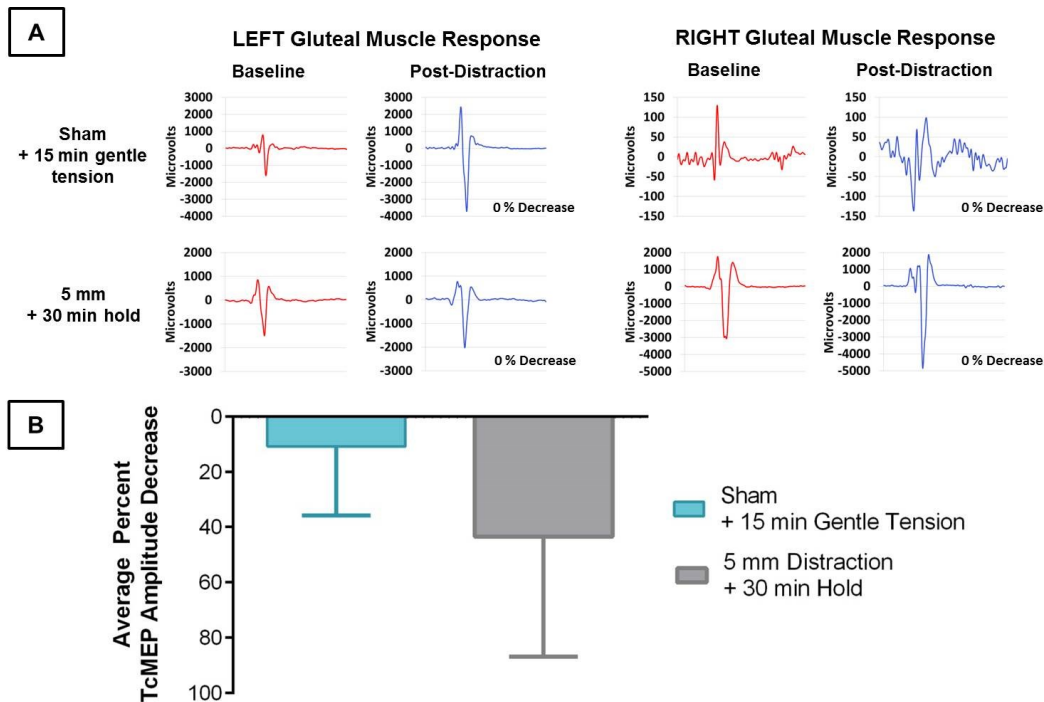
Figure 1 TcMEP amplitude is not significantly affected following a 30 minute prolonged distraction. (A) Representative TcMEP recordings distal to the injury site. (B) Quantification of average percent TcMEP amplitude decrease following injury. Motor-evoked potentials recorded distal to the injury site are not significantly affected following a 30 minute prolonged 5-mm distraction injury. Data represent the average and standard deviation (n = 6 and 3 per sham and 30 min prolonged distraction groups, respectively).

Behavioral assessment, on the other hand, confirmed a motor deficit following a 30 minute prolonged distraction. While mechanoceptive response does not significantly change following a 30 minute prolonged distraction injury for one week compared to sham/baseline (Figure 2), a 30 minute prolonged distraction injury does result in a significant motor deficit for one week post-injury compared to sham/baseline, as shown in Figure 3A. The individual BBB scores for animals that underwent sham and 30 minute prolonged distraction injuries are shown in Figures 3B and 3C, respectively. The high variability following a 30 minute prolonged distraction was due to the occurrence of dislocation in one of the animals. Specifically, two of the three animals that underwent a 30 minute prolonged distraction injury exhibited hindlimb

rotation and, thus, had a BBB score of 18 immediately following injury. The third animal, however, experienced dislocation and had a BBB score of 0 immediately following injury.
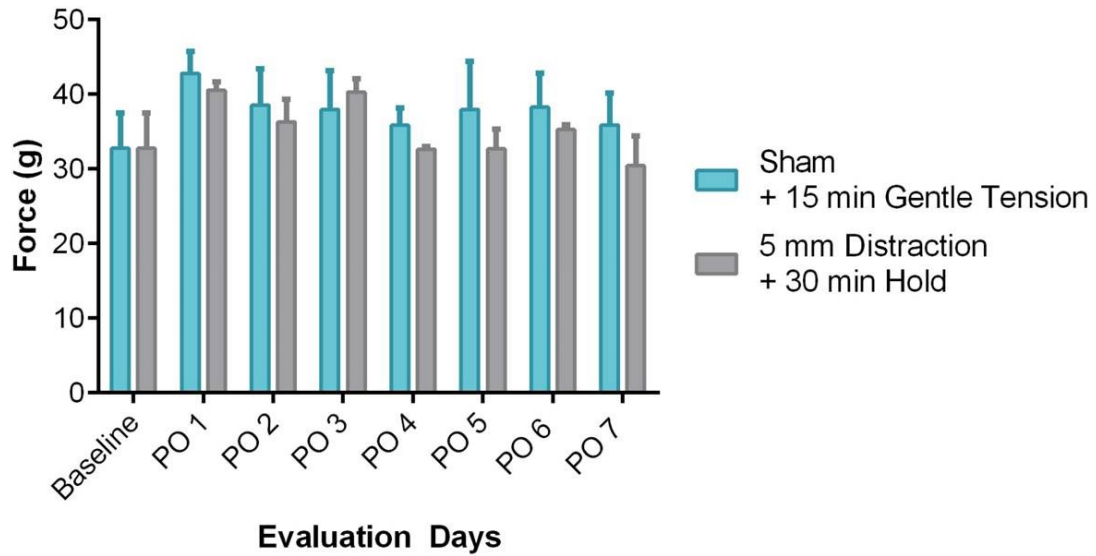
Figure 2 Mechanoceptive response is not significantly affected following 30 minute prolonged distraction. Data represent the average and standard deviation (n = 6 and 3 per sham and 30 min prolonged distraction groups, respectively).
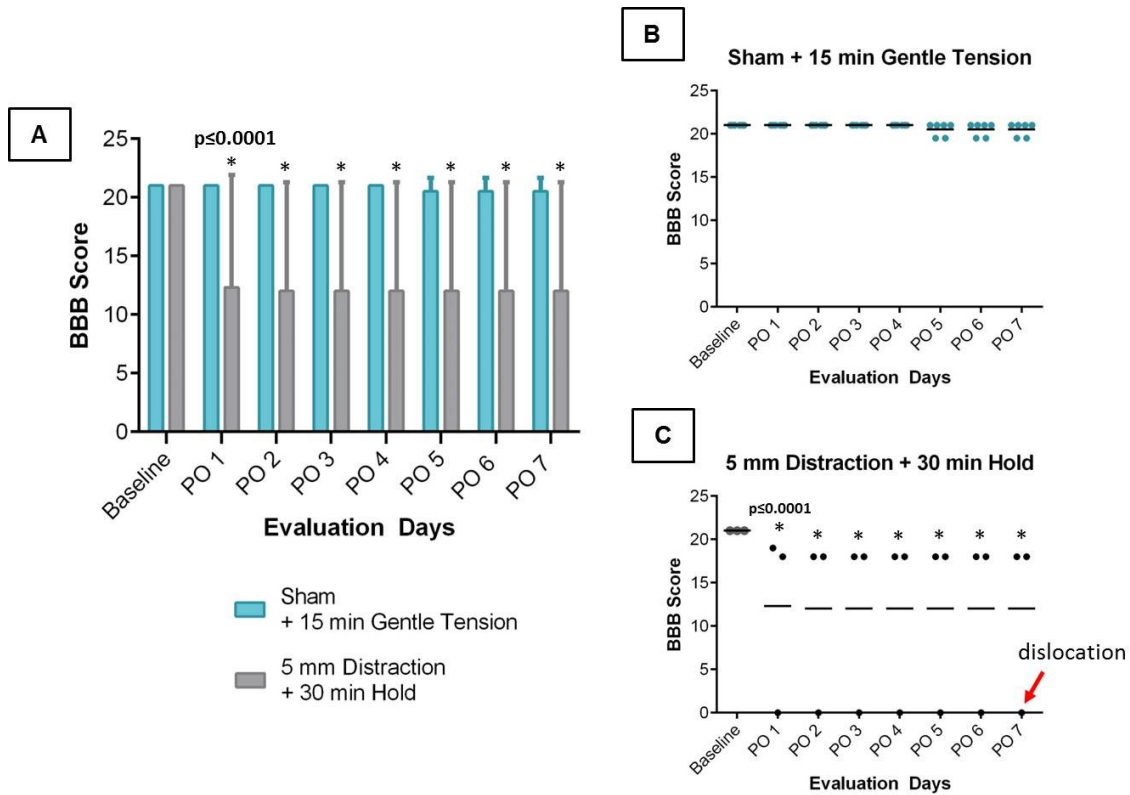
Figure 3 Motor function is impaired following 30 minute prolonged distraction. Evaluation of BBB locomotor rating score over seven days following a 30 minute prolonged 5-mm distraction revealed a significant motor deficit. Data represent the average and standard deviation (n = 6 and 3 per sham and 30 min prolonged distraction groups, respectively). *p≤0.0001 compared to sham/baseline.

Consistent with the observed mild functional deficit, histological analysis revealed that the structural integrity of the spinal cord was not compromised following a 30 minute prolonged distraction. Specifically, no tissue loss was detected in the H&E stained spinal cord sections (Figure 4). In addition, neuron-depleted regions (indicated by NeuN) and positive staining for GFAP and ED1 were not visualized in the spinal cord following injury (Figure 4). These results indicate that reactive gliosis and macrophage activation, known effectors of secondary injury cascades, are absent in this mild injury paradigm.

	All cells	Neurons	Reactive astrocytes	Activated macrophages
	H&E	**NeuN**	**GFAP**	**ED1**

Sham + 15 min Gentle Tension

5 mm + 30 min Hold

Scale bar = 500 μm
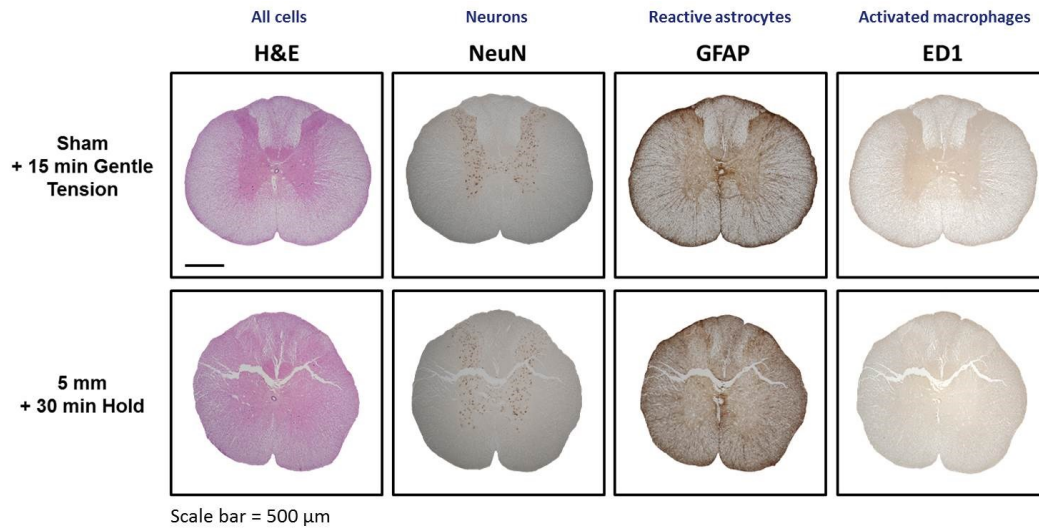
Figure 4 Thirty minute prolonged distraction does not cause spinal cord damage. Transverse spinal cord sections taken from the epicenter of sham and 30 minute prolonged distraction animals and stained for H&E, NeuN, GFAP, and ED1 revealed intact spinal cord tissue and lack of positive staining for reactive astrocytes and activated macrophages.
Scale bar = 500 μm.

Quantification of the intensity distribution along a straight line across each H&E-stained spinal cord section revealed that the average signal intensity due to tissue damage in the 7-mm distraction group from the previous study was significantly higher than the average signal intensities in the sham and prolonged distraction groups, as shown in Figure 5. Conversely, a significant difference between the average signal intensities in the sham and 30 minute prolonged distraction groups was not observed, thereby confirming that a 30 minute prolonged 5-mm distraction does not induce mechanical damage to the spinal cord tissue.
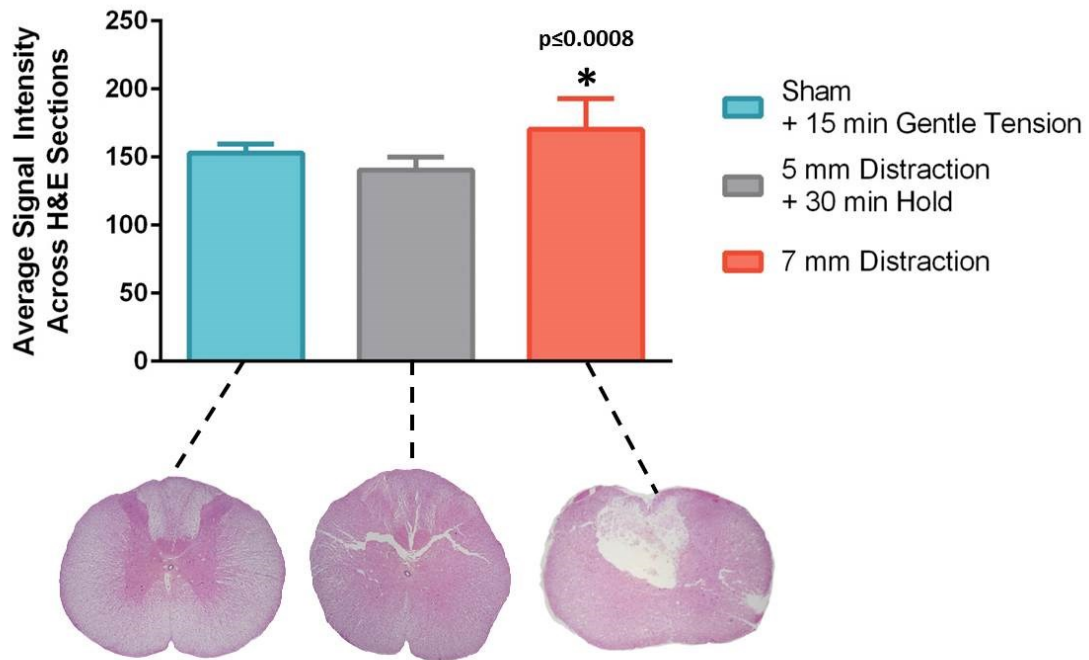
Figure 5 Absence of observable tissue loss following 30 minute prolonged distraction. Quantification of average signal intensity across H&E-stained spinal cord sections confirmed a lack of mechanical damage to the spinal cord tissue following a 30 minute prolonged distraction. While tissue damage in animals distracted 7-mm results in significantly higher average signal intensity, the average signal intensities in the sham and prolonged distraction groups are not significantly different. Data represent the average and standard deviation (n = 6 and 3 per sham and 30 min prolonged distraction groups, respectively). *p≤0.0008 compared to sham/30 minute prolonged distraction.

The lack of tissue loss, neuron-depleted regions, and positive staining for GFAP and ED1 observed at the epicenter was also observed in proximal and distal sections following a 30 minute prolonged distraction, as shown in Figures 6, 7, 8, and 9.

	Proximal	**Epicenter**	**Distal**
Sham + 15 min Gentle Tension			
5mm + 30 min Hold			
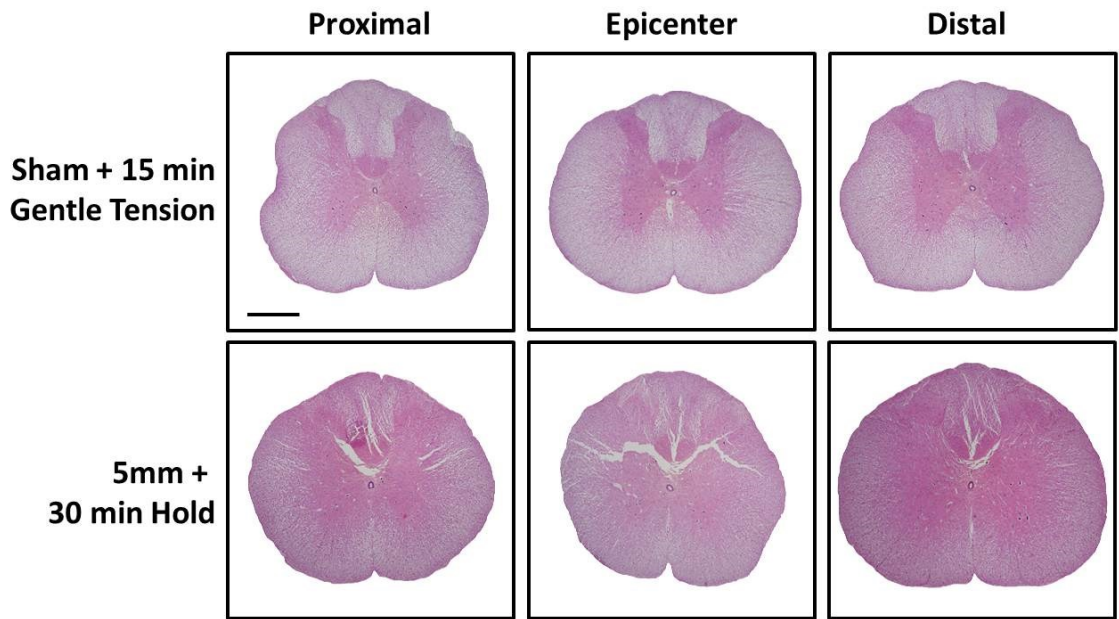
Figure 6 H&E-stained spinal cord sections following sham injury and 30 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 30 minute prolonged 5-mm distraction animals stained with H&E reveal intact spinal cord tissue. Scale bar = 500 μm.

	Proximal	**Epicenter**	**Distal**
Sham + 15 min Gentle Tension			
5mm + 30 min Hold			
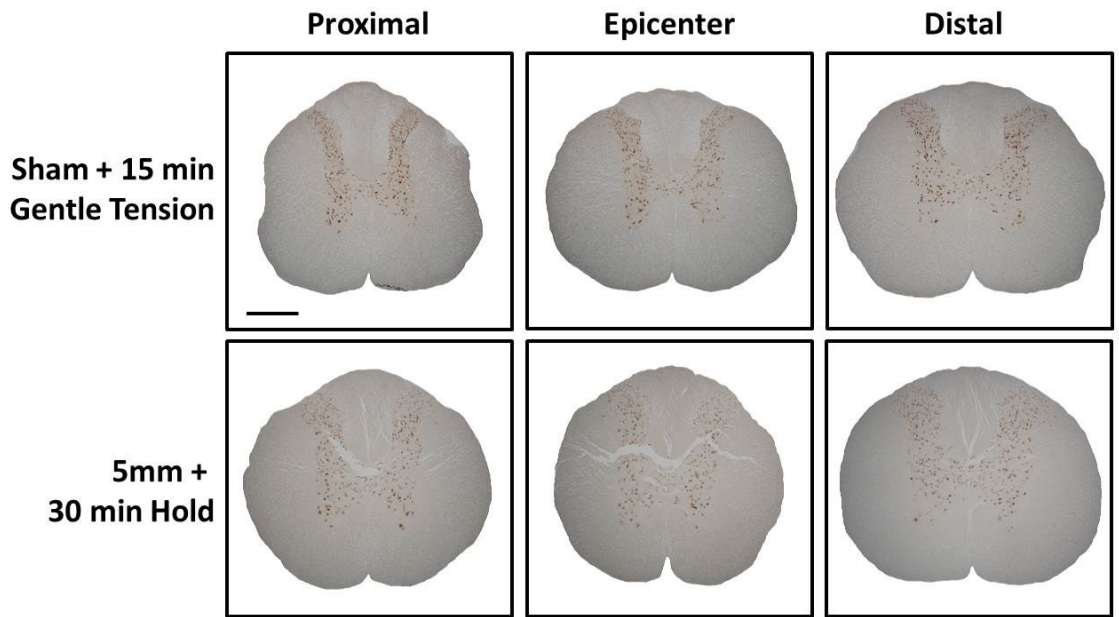
Figure 7 NeuN-stained spinal cord sections following sham injury and 30 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 30 minute prolonged 5-mm distraction animals stained with NeuN reveal a lack of neuron-depleted regions. Scale bar = 500 μm.
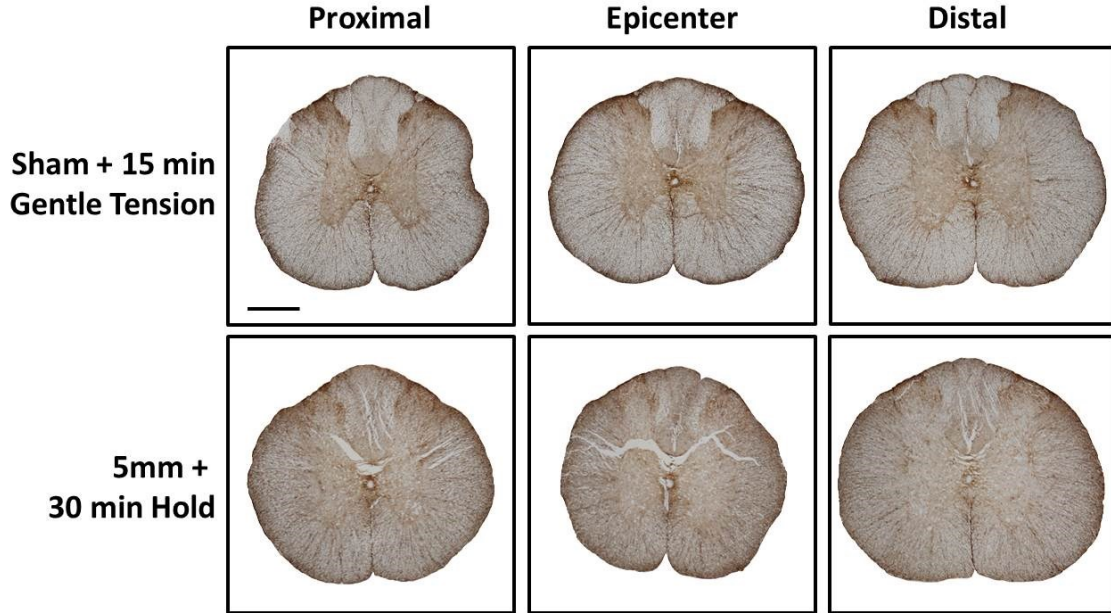
Figure 8 GFAP-stained spinal cord sections following sham injury and 30 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 30 minute prolonged 5-mm distraction animals stained with GFAP reveal no increase in reactive astrocytes compared to sham. Scale bar = 500 μm.
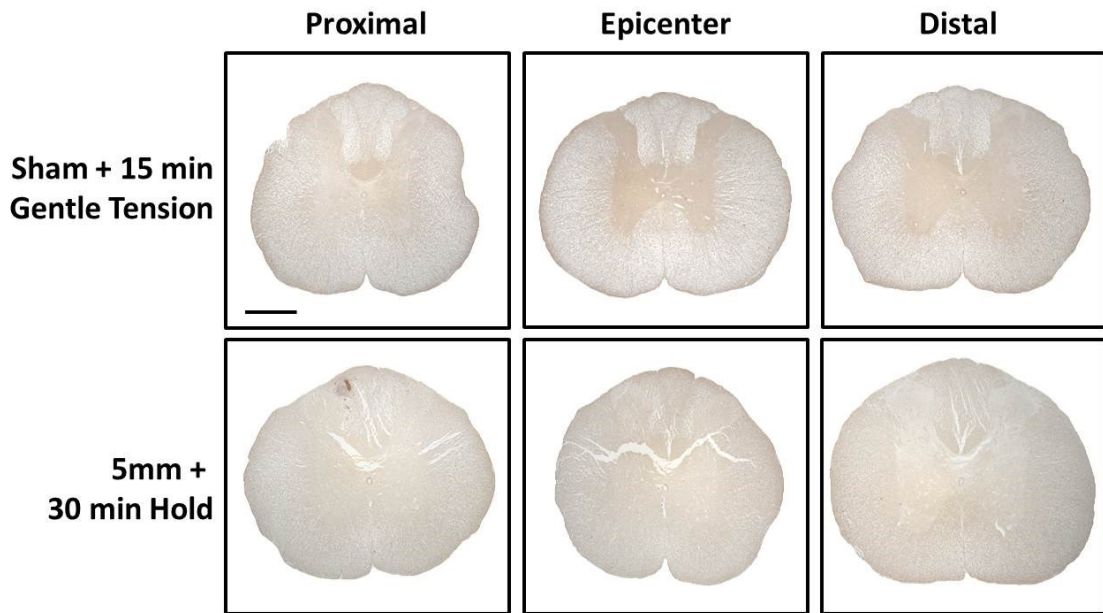
Figure 9 ED1-stained spinal cord sections following sham injury and 30 minute prolonged distraction. Transverse spinal cord sections taken from proximal, epicenter, and distal regions of sham and 30 minute prolonged 5-mm distraction animals stained with ED1 reveal a lack of positive staining for activated macrophages. Scale bar = 500 μm.

We also evaluated the extent of hypoxic insult following a 30 min prolonged distraction injury in one animal as a preliminary step to assess the effect of time held in a distracted position on the severity of the vascular compromise. The resulting trace is shown in Figure 10. Similar to the hypoxic insult detected in response to a 15 minute prolonged distraction, a 30 minute prolonged distraction resulted in an immediate, transient sharp decline in pO_2 levels followed by a mild hypoxic insult for the duration of time held in the distracted position. Thus, the severity of the hypoxic insult was not exacerbated by the extended period of time (30 minutes) in the distracted position.
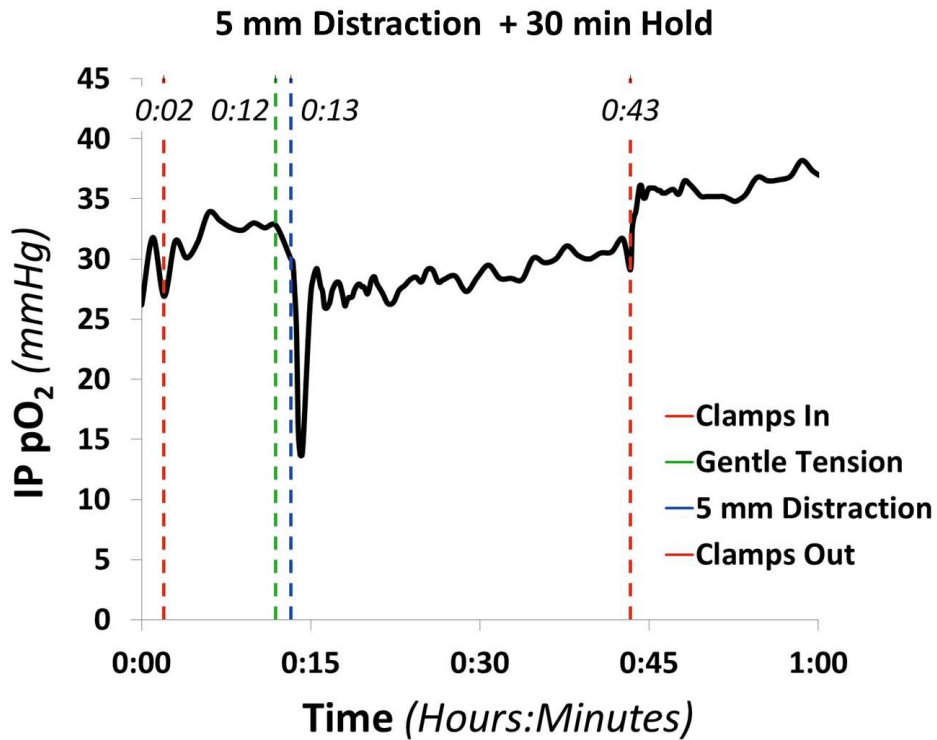
Figure 10 Representative pO_2 recording distal to the injury site following 30 min prolonged distraction.

In summary, the results obtained in this preliminary study indicate that a longer time in the distracted position does not exacerbate the injury severity. Further studies are warranted to validate these results.

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BIOGRAPHICAL INFORMATION

Jennifer E. S. Bell received her bachelor in science degree in Chemistry in 2006 from Southern Methodist University in Dallas, TX. She worked in a biochemistry laboratory at the University of Texas Southwestern Medical Center before entering graduate school in the fall of 2008. Since joining the Regenerative Neurobiology Laboratory at The University of Texas at Arlington under the supervision of Dr. Mario I. Romero-Ortega, her doctoral research has involved the establishment and characterization of an in vivo model of distraction spinal cord injury. Through this research project, she has had the privilege of working in collaboration with clinicians at Texas Scottish Rite Hospital for Children and has gained a deeper appreciation for the impact of this research on children undergoing scoliosis corrective surgery. Jennifer has presented her work at four national meetings. While at UTA, she has co-authored two peer-reviewed publications and has authored a third manuscript which is in the process of being submitted. In the spring of 2012, she was honored with The Alfred R. and Janet H. Potvin Outstanding Bioengineering Student Award. Throughout her graduate career, Jennifer has developed a love of teaching, a passion she wishes to pursue following graduation. She currently lives in Dallas, TX with her husband, Brad Bell, and they are expecting their first child.