PHARMACOLOGICAL NEUROPROTECTION

IN

CERVICAL SPINAL CORD INJURY

by

Jae Ho Lee

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Abstract

Spinal cord injury (SCI) is a devastating condition that causes paralysis below the level of the injury. To date, there is no convincingly effective treatment. An enormous preclinical and clinical effort is underway to find a treatment, and one approach is to search for pharmacological agents that are already in clinical use (albeit for different indications), but that may also have neuroprotective properties. Examples of such drugs are magnesium, Riluzole (sodium channel blocker), minocycline and statins.

While the majority of human SCI occur in the cervical spinal cord, the vast majority of laboratory SCI research employs animal models of thoracic SCI. An important step, therefore, in the preclinical evaluation of novel treatments is to assess their efficacy in a model of cervical SCI. First, I describe the development of a novel unilateral contusive model of cervical SCI with refined biomechanical, functional, and histological parameters using the Infinite Horizon spinal cord injury device. I conducted a series of experiments in which the spinal cord was injured using various impact forces, impact trajectories, and impact locations off the midline. Behavioral deficits were assessed using a variety of forelimb function tests, after which the cords were evaluated histologically. From these series of experiments, I established a new cervical unilateral spinal cord injury contusion model.

Next, I evaluated the neuroprotective effects of minocycline and simvastatin in the clinically relevant unilateral cervical contusion model. Minocycline is a commonly prescribed tetracycline antibiotic that is prescribed for acne. Simvastatin is one of many hydroxymethylglutaryl-coenzyme-A reductase inhibitors that lower cholesterol. As both drugs have translational potential and have been reported to have neuroprotective properties in various neurological diseases, I assessed the neuroprotective effects of these drugs using a host of functional and histological assessments. In the end, there were no neurological improvements with minocycline or simvastatin treatment after a cervical contusion injury.
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Co-authorship Statement

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My role in the described set of experiments was to perform them with the aid of Jie Liu and Anthea M.T. Stammers for animal surgeries, and with the aid of Elena Okon for behavioral analyses. Surgeries, behavior assessments, histology, data analyses, and manuscript preparation was performed by myself, under the guidance of my graduate supervisor Dr Brian K. Kwon.
CHAPTER 1

Introduction
1.1 Introduction

Spinal cord injury (SCI) is a devastating disease that results in paralysis below the level of the injury. The first documented case of SCI was in the ancient Egyptian medical papyrus around 2500 years B.C. (Hughes, 1988) A condition with a crushed vertebra in the neck was characterized as an “ailment not to be treated” that had symptoms described as loss of motion, sensation and bladder control below the level of injury. It was not until around 450-380 B.C. when Hippocrates’ first attempt to treat SCI was documented, in which he utilized dietary interventions consisting of “4-9 pints of donkey milk combined with honey and a special mild white wine from Mendez in Egypt” (Marketos and Skiadas, 1999). In addition, he described some of the secondary conditions that are associated with paralysis such as constipation, dysuria, pressure sores and edema (Lifshutz and Colohan, 2004; Anderberg et al., 2007). In the 1900s, Santiago Ramon J. Cajal described the structure of the nervous system which lead to a formulation of the neuron doctrine (de Carlos and Borrell, 2007). Reginald Allen introduced the first standardized weight drop device revolutionizing SCI research (Allen, 1911; Lifshutz and Colohan, 2004). In the First World War era, Sir Ludwig Guttmann introduced rehabilitation treatments to SCI patients (Anderberg et al., 2007).

SCI has enormous social and economical impact. Recent estimates suggest that there are close to 1.5 million people in North America with SCI, more than three times than previously thought (Christopher and Dana Reeve Foundation, 2009). In the past, SCI was perceived to affect mostly the young and healthy individuals in the age group between 25 and 35 years old, but now we are aware that there is actually a bimodal distribution of individuals with SCI, with an additional age group suffering from SCI between 60 to 65 years old (Sekhon and Fehlings, 2001; van den Berg et al., 2010). It is estimated that it costs up to 3.2 million dollars in a life time of an individual with high tetraplegia and costs close to 40 billion dollars annually for the health care system (Christopher and Dana Reeve Foundation, 2009; NSCISC, 2010). More than 60 % of SCI are at the cervical level and most (60 %) SCI are incomplete (NSCISC, 2010).

Since the First World War, there has been a dramatic improvement in the medical management of patient with SCI (Anderberg et al., 2007), but to date, there are still no convincingly efficacious treatments to improve neurologic outcome after acute human SCI. To date, methylprednisolone is the only treatment that is widely used; however, due to concerns regarding its safety and controversy surrounding its therapeutic effects, its use has been abandoned
Without a question, there is a dire need for an effective treatment for SCI. For this reason, scientific researchers are extensively investigating numerous potential therapies that have been shown to improve functional and histological outcomes after animal models of SCI, with the hope of advancing to clinical trials and ultimately treating SCI. Some of the potential treatments that are currently in clinical trials include Riluzole (sodium channel blocker) (ClinicalTrials.gov Identifier: NCT00876889), Cethrin (Rho GTPase inhibitor) (ClinicalTrials.gov Identifier: NCT00610337), anti-NOGO (ClinicalTrials.gov Identifier: NCT00406016) and minocycline (ClinicalTrials.gov Identifier: NCT00559494).

There are many causes of SCI. More than 75% of SCI are caused by a blunt traumatic impact from motor vehicle accidents, falls, work accidents and sports/recreation accidents (NSCISC, 2010; Christopher and Dana Reeve foundation, 2009). SCI progresses in two stages: primary injury followed by secondary injury. Primary injury consists of the initial mechanical damage to the neurons and glial cells. Secondary injury is the expansion of the primary injury by a cascade of multiple complex intertwined mechanisms.

1.1.1 Primary Injury

SCI is typically instigated when the spinal column fractures and/or dislocates, imparting a combination of contusive, compressive, and distractive forces on the cord, which leads to shearing of the cellular membrane and disruption of the blood spinal cord barrier (BSB). The mechanical force applied to the cell bodies, blood vessels and axons along the spinal cord physically disrupts the integrity of the spinal cord upsetting ionic balance that affects the electrical signals needed for neurological function below the injured level (Beattie et al., 2002). Immediately after the insult, from the breach of the BSB, blood starts to seep into the surrounding epidural, subdural, subarachnoid and intramedullar spaces. As blood is toxic to the central nervous system (CNS), the extravasation of blood has a detrimental impact (Asano, 1980). The area of hemorrhage corresponds to the size of the cavity formed in later stages of secondary injury (Noble and Wrathall, 1989a; Noble and Wrathall, 1989b). In addition, blood vessels constrict that limit blood flow to the tissues and secondary injury commences (Tator, 1991; Young et al., 2002).
1.1.2 Secondary Injury

Secondary injury is a cascade of events that lead to the expansion of the primary injury. The extent of damage inflicted by primary injury has enormous impact on secondary injury development. A number of pathophysiological processes are triggered by the initial mechanical injury leading to prolonged secondary injury phase, which begins at the time of injury and can last days to months (Fleming et al., 2006; Hall and Springer, 2004; Norenberg et al., 2004; Onose et al., 2009; Profyris et al., 2004).

Immediately after the BSB disruption, hemorrhage starts to slowly expand from the site of trauma (Tator, 1995; Tator and Fehlings, 1991). The penumbra and edema surrounding the injury site is formed around 6 hours (Guth et al., 1999). The area of edema and hemorrhage continues to enlarge, white and grey matter definition is lost, and inflammation and oxidative stress commence (Tator, 1995). By 1 week, hemorrhage disappears and the lesion site is filled with invaded cells and cellular debris (Beattie et al., 2002). At 3 weeks post-injury, cystic regions are formed along with scar tissue, mainly originated from glial cells that surround the clear visible cavitation (Beattie et al., 2002). The cystic region expands for considerable distances both rostral and caudal from the primary injury. Necrotic cell death occurs immediately after the primary injury and persists, while apoptosis is initiated about 1 day after trauma. Wallerian degeneration, degeneration of injured axons, starts both rostrally and caudally after the first day and continues chronically (Tator, 1995).
1.2 The Pathophysiology of Secondary Injury

The initial mechanical insult and vascular disruption is followed by a complex series of cascading events that includes excitotoxicity, oxidative stress, inflammation, and cell death. Mechanical damage leading to the release of intracellular contents such as excitatory amino acid neurotransmitters and recruitment of intrinsic (residential microglial cells and astrocytes) and peripherally derived cells (hematogenous neutrophils, macrophages, lymphocytes and natural killer cells) mediate these molecular events of secondary injury. The immune responses mediated by the peripheral cells are further divided into an innate and adaptive response. The innate immune cells: neutrophils, hematogenous macrophages, natural killer cells respond to infection and inflammatory stimuli and in turn recruit B and T lymphocytes of the adaptive immune system. The detailed mechanisms of how secondary injury progresses are still an active area of research.

1.2.1 Excitotoxicity

Glutamate is a major excitatory neurotransmitter in adult central nervous system. Under normal physiological conditions, glutamate is released from the presynaptic membrane by Ca\(^{2+}\) mediated exocytosis. Once released, glutamate binds to receptors on the postsynaptic membrane. There are two major types of glutamate receptors: (i) ionotropic receptors, which lead to the opening of ion channels and (ii) metabotropic receptors that indirectly activate ion-channels on the plasma membrane through a G protein mediated signaling cascade. The ionotropic receptors include N methyl-d-aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Birch et al., 1988; Danysz et al., 1989; Faden and Simon, 1988; Li and Tator, 1999; Liu et al., 1997; Wrathall et al., 1994). The metabotropic glutamate receptors are divided into three groups group I, II and III based on receptor structure and physiological activity (Doble, 1999; Mills et al., 2001a). The concentration of glutamate in the synaptic cleft is tightly regulated by Na\(^+\) dependent transporters such as Glt1, GLAST and EAAC1 on the presynaptic membrane and glial cells (Li et al., 1999; Nicholls and Attwell, 1990; Roettger and Lipton, 1996). Excitotoxicity occurs when increased levels of glutamate over-stimulate the glutamatergic receptors that lead to destruction of cells.
After SCI, the extracellular increase of glutamate displays temporal and spatial distribution. Glutamate levels in both the white and grey matter increase as early as 15 minutes and up to 1 hour after injury (Liu et al., 1991). During this period, extracellular glutamate levels increase 10 fold and return back to baseline level by 1 hour (Liu et al., 1991; Liu et al., 1999; Xu et al., 2004). Glutamate release is localized within only a few millimeters of the site of injury, while there are minor increases in glutamate levels rostral and caudal to the injury (McAdoo et al., 1999; Xu et al., 2004). The rise in extracellular glutamate can occur due to BSB disruption, exocytosis, reverse glutamate transport, cell lysis and secretion by the invaded peripheral cells (McAdoo et al., 2000; McAdoo et al., 2005; Roettger and Lipton, 1996; Rothstein et al., 1996; Tanaka et al., 1997; Vera-Portocarrero et al., 2002).

The current understanding of glutamate mediated excitotoxicity comes mainly from pharmacological studies using various agonists and antagonists of glutamate receptors. The detrimental effects of excess extracellular glutamate to neurons can be demonstrated when glutamate is directly delivered into a normal spinal cord, where the number of neuronal cell bodies decreased dramatically by 25% (Liu et al., 1999). In the grey matter, excess extracellular glutamate over activates both ionotropic and metabotropic receptors. This prolonged activation of ionotropic receptors allows nonspecific cations to flow in causing further ionic imbalance and intracellular Ca^{2+} overload. When NMDA is continuously delivered to a normal spinal cord there is a dose dependent damage (Nag and Riopelle, 1990). Similarly, agonists of AMPA and kainite receptors quisqualic acid and kainic acid produce excitotoxic injury that is similar to traumatic SCI (Pisharodi and Nauta, 1985). On the other hand, when the NMDA receptors are blocked by applying a selective antagonist, dizocilpine (MK801), it is shown to improve neurological outcomes after SCI (Faden and Simon, 1988; Gomez-Pinilla et al., 1989; Li and Tator, 1999). Administering 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(f) quinoxaline (NBQX), a highly selective antagonist of AMPA and kainite receptors, prior to injury reduced hindlimb deficits and improved histological outcomes in a dose dependent manner (Follesa et al., 1998; Wrathall et al., 1992; Wrathall et al., 1994). Prolonged activation of metabotropic receptor, group-I mGluRs also results in elevation of the intracellular Ca^{2+} levels, but in addition, group-I mGluRs further enhance glutamate release by a protein kinase C mediated inhibition of presynaptic K+ channels (Choi, 1992; Pin and Duvoisin, 1995). Application of a group I GluRs agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid
(ACPD) has neurotoxic effects (McDonald et al., 1993; McDonald and Schoepp, 1992; Sacaan and Schoepp, 1992), whereas selective antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA) decreases extracellular glutamate concentrations (Mills et al., 2000; Mills et al., 2001a). In contrast, activating group-II and -III GluRs may prevent cell death (Allen et al., 1999; Bruno et al., 1994). Applying group III agonist, 1-2-amino-4-phosphonobutyric acid (l-AP4) reduces extracellular glutamate levels. An antagonist of group-II metabotropic receptors (aS)-a-amino-a-[(1S,2S)-2-carboxycyclopropyl]-9Hxanthine-9-propanoic acid (LY 341495), in contrast increases extracellular glutamate concentration (Mills et al., 2001b). These studies indicate that activating both ionotropic and metabotropic receptors contributes to the secondary injury progression and there may be a synergistic effect between the two types of glutamate receptors that mediates excitotoxicity (Alagarsamy et al., 1999; Gereau and Heinemann, 1998).

Elevation of extracellular glutamate occurs by different mechanisms in the white matter (Ouardouz et al., 2009; Park et al., 2004). Glutamate is released in the extracellular compartment by membrane damage, necrotic and lysed neurons and Na⁺ mediated reverse glutamate transport (Park et al., 2004). Excitotoxicity in the white matter causes destruction of both axons and oligodendrocytes (Ouardouz et al., 2009). Axons, astrocytes and oligodendrocytes all express ionotropic glutamate receptors (Karadottir et al., 2005; Li and Stys, 2000; McDonald et al., 1998; Ouardouz et al., 2009; Salter and Fern, 2008). Originally it was thought that glial cells only expressed non-NMDA receptors, but NMDA receptors have also been discovered (Alagarsamy et al., 1999; Gereau and Heinemann, 1998). However, the role of these NMDA receptors on the oligodendrocytes are somewhat puzzling as administration of NMDA antagonist has no effect on reducing dorsal column injury (Agrawal and Fehlings, 1997; Li et al., 1999; Ouardouz et al., 2006). On the other hand, NBQX reduces oligodendrocyte damage in a dose dependent manner (Xu et al., 2004). This shows the involvement of AMPA/kainite receptors on myelin destruction after SCI. In addition to the destruction of myelin, axons are also damaged by glutamate as well. Recently it was shown that axons express kainate receptors (Ouardouz et al., 2009). The ionic imbalance caused by SCI reverses the influx of glutamate transporters increasing the extracellular glutamate levels (Li et al., 1999). Excess extracellular glutamate activates AMPA and kainite receptors on the axonal membrane that leads to a drastic increase of intracellular Ca²⁺ levels (Ouardouz et al., 2009).
Glutamate mediated excitotoxicity of both grey and white matter leads to dramatic intracellular \(\text{Ca}^{2+}\) increase. Elevated intracellular \(\text{Ca}^{2+}\) triggers phospholipase and lipase activation that leads to increased oxidative stress and cell death (see below) (Anderson and Stokes, 1992; Stokes et al., 1983).

### 1.2.2 Oxidative Stress

Oxidative stress occurs when the level of reactive oxygen species (ROS) exceed the capability of enzymes that can neutralize their reactivity. In the normal physiological state, nitric oxide (NO) and free radicals such as superoxide (\(\text{O}_2^-\)) and hydroxyl radical (\(\text{OH}^-\)) are by-products of normal cellular and biochemical processes that use oxygen (Coyle and Puttfarcken, 1993). Superoxide is a common precursor for ROS (Liu et al., 1998). Excess superoxide anion can react with NO to form peroxynitrite (\(\text{ONOO}^-\)) (Dawson, 1994; Liu et al., 2000; Liu et al., 2006; Scott et al., 1999). The majority of superoxide anions (\(\text{O}_2^-\)) are produced from the electron transport chain of mitochondria (Liu et al., 1998).

The free radical scavengers such as superoxide dismutase (SOD), glutathione peroxidase and catalase convert free radicals into \(\text{O}_2\) or water (Azbill et al., 1997; Dawson et al., 1993; Liu et al., 1998). In addition, antioxidants: ascorbic acid, glutathione and vitamin E bind to free radicals to neutralize their damaging properties (Liu et al., 1998). SOD converts superoxide into hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and glutathione peroxidase and catalase reduce \(\text{H}_2\text{O}_2\) to \(\text{H}_2\text{O}\) (Liu et al., 1998; Lucas et al., 2002). Under normal conditions, there is a dynamic equilibrium between the formation and neutralization of free radicals (Liu et al., 1998).

Following SCI, NO, superoxide anion and iron levels are increased and free radical formations are accelerated (Christie et al., 2008; Dawson, 1994; Liu et al., 1998; Liu et al., 2000; Liu et al., 2004). Peroxynitrite formation results in marked neuronal loss and locomotor dysfunction after SCI (Bao and Liu, 2002). The increase in NO production depends on intracellular \(\text{Ca}^{2+}\) levels. Increased intracellular \(\text{Ca}^{2+}\) levels from glutamate stimulation increase nitric oxide synthase (NOS) activity and produce excess NO. Glutamate induced excitotoxicity is reduced with a NOS inhibitor (Dawson, 1994). Elevated intracellular \(\text{Ca}^{2+}\) levels trigger membrane phospholipase and lipase activation, mitochondria dysfunction and initiation of protease (Anderson and Stokes, 1992; Happel et al., 1981; Moriya et al., 1994; Stokes et al., 1983). Phospholipase and
lipase activation releases free fatty acids and arachidonic acid (Faden et al., 1987). Excess superoxide anion is produced from mitochondria damage (Azbill et al., 1997). As early as 1 hour after SCI, mitochondria activity is decreased by 23% and by 24 hours 34% compared to control animals. Four hours after injury, reduced mitochondria activity is followed by the generation of ROS (Azbill et al., 1997). Excessive $\text{O}_2^-$ and iron can also produce ROS. Iron can bind to $\text{H}_2\text{O}_2$ and produce hydroxyl radical (Liu et al., 1998). Iron levels are increased by the injury itself. Intracellular iron is released by injured cells and hemorrhage from the primary injury releases heme (Liu et al., 1998; Mautes et al., 1998). Heme released from hemorrhage increases hemeoxygenase (HO) expression that converts heme to iron, biliverdin and carbon monoxide. This conversion further elevates the iron levels (Liu et al., 1998; Mautes et al., 1998; Sharma et al., 2000).

Increased ROS cause lipid peroxidation, protein peroxidation and damage of DNA by binding to nucleic acids (Joosten and Houweling, 2004). The upregulation of ROS can bind and take a proton from a polyunsaturated fatty acid of free fatty acids and membrane forming additional free radicals and causing lipid peroxidation (Lucas et al., 2002). This interaction causes changes in membrane fluidity, permeability, altered function of membrane-associated proteins, and increased likelihood of organelle and cell lysis. Lipid peroxidation produces malondialdehyde (MDA) and 4-hydroxy-2-trans-nonenal (4-HNE) (Lucas et al., 2002). There are two waves of lipid peroxidation as measured by a lipid peroxidation marker, Malondialdehyde (MDA). The first wave of MDA production starts from 1 to 6 hours and the second wave starts from 24 hours to 120 hours (Christie et al., 2008). As mentioned above, the first wave of lipid peroxidation is a result of glutamate mediated excitotoxicity. The second wave appears when neutrophils and microglial cells are infiltrating the injury site. Infiltrated neutrophils, activated microglial cells and macrophages also release free radicals as a by-product of phagocytosis (Joosten and Houweling, 2004). The timing of the second wave of lipid peroxidation suggests that oxidative stress is also involved with inflammation.

Further evidence indicating a contribution of oxidative stress to secondary injury comes from application of antioxidants and antioxidative drugs. Treatments with vitamin E, melatonin and selenium, or non-glucocorticoid 21- aminosteroid tirilazad mesylate (U-74006F) and methyprednisolone applied after SCI, reduce fatty acid production, prostanoids and trauma induced cholesterol loss (az-Ruiz et al., 2000; Fujimoto et al., 2000; Iwasa et al., 1989; Kaptanoglu et al., 2000; Koc et al., 1999; Saunders et al., 1987; Taoka et al., 2001).
1.2.3 Inflammation

After SCI, hematogenous neutrophils are first to respond by rapidly entering the injury site (Kwon et al., 2004). Neutrophils migrate into the lesion site and secrete lytic enzymes, reactive oxygen species and cytokines that lead to inflammation and increased recruitment of more peripheral cells (Bao and Liu, 2002; Chandler et al., 1995; Chao et al., 2005; Kwon et al., 2004; Liu et al., 2006; Merrill et al., 1993; Neumann, 2001; Shamash et al., 2002). Neutrophil levels measured by myeloperoxidase (MPO) activity are elevated as early as 3 hours and up to 3 days post-injury (Carlson et al., 1998; Chatzipanteli et al., 2000; Taoka and Okajima, 1998). Experimental therapies that diminish neutrophils provide neuroprotection and improve neurological recovery after SCI (Eng and Lee, 2003; Giulian and Robertson, 1990; Gok et al., 2007; Gris et al., 2004; Noble et al., 2002; Taoka et al., 1997). After SCI, resident microglial cells are activated and hematogenous monocytes, macrophages and lymphocytes infiltrate the injured spinal cord (Chan, 2008; Donnelly and Popovich, 2008). Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α released from the neutrophils and damaged cells which attract macrophages, lymphocytes and microglial cells (Donnelly and Popovich, 2008; Klusman and Schwab, 1997; Schnell et al., 1999). Microglial cells are activated and transformed morphologically to express specific cell adhesion molecules and secret additional TNF-α and IL-1β to recruit more peripheral cells (Bethea et al., 1999; Hausmann, 2003; Hermann et al., 2001; Pearse et al., 2004; Schnell et al., 1999). There are different populations of macrophages: bone marrow derived and peritoneal macrophages (Blight, 1994; Longbrake et al., 2007; Popovich et al., 1999). Once penetrated, macrophages and lymphocytes persist in the spinal cord for weeks and months after injury (Popovich et al., 2003). They also recruit additional microglial and macrophages, and during phagocytosis produce free radicals, glutamate, lysosomal and proteolytic enzymes that contribute to further expansion of secondary injury (Bethea et al., 1999; Popovich et al., 2003). However, a potentially beneficial role of the inflammatory process has also been reported. After injury, macrophages display time dependent functions. Inhibiting early macrophage activation has been shown to be neuroprotective and improve function recovery in rodent SCI (Blight, 1994; Eng and Lee, 2003; Giulian and Robertson, 1990; Gok et al., 2007; Noble et al., 2002; Popovich et al., 1999), where as activating macrophages in the later stages of secondary injury promotes recovery, axonal regeneration and oligodendrocyte maturation (Gensel et al., 2009; Schonberg et al., 2007).
In vitro experiments suggest that once the macrophages occupy the lesion site, cues from the microenvironment determine whether macrophages will become a M1 or M2 type. It is the M1 macrophages that can produce oxidative metabolites and produce more pro-inflammatory cytokines (Sica et al., 2006). At later stages of secondary injury, M2 type macrophages promote recovery by angiogenesis, matrix remodelling and suppressing the destructive immune response (Sica et al., 2006).

As mentioned, invading neutrophils, macrophages and activated microglial cells produce cytokines. Not all cytokines promote inflammation, TNF-α, IL-1β, IL-6, IL-8 and IL-12 are pro-inflammatory and IL-10 is anti-inflammatory (Bethea et al., 1999; Brewer et al., 1999; Donnelly and Popovich, 2008; Klusman and Schwab, 1997).

TNF-α is a proinflammatory cytokine that is normally secreted at low levels by neurons (Xu et al., 1998; Yan et al., 2001). TNF-α is secreted by neutrophils, macrophages, oligodendrocytes and reactive microglial cells (Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Lacroix et al., 2002; Okada et al., 2004; Yan et al., 2001). TNF-α induces IL-1 and IL-6 and attracts more macrophages and microglial cells by activating NF-kB, an inducible transcription factor, that regulates expression of proinflammatory genes, cellular adhesive molecules, inducible nitric oxide synthase and interleukins that exacerbate inflammatory activity (Xu et al., 1998). Similar to macrophages, TNF-α can be detrimental or beneficial at different stages of secondary injury. Almost immediately after trauma, TNF-α levels and TNF- receptors expressions are elevated (Bartholdi and Schwab, 1997; Pan et al., 1997; Wang et al., 1996; Yan et al., 2003). Administering TNF-α 1 day after SCI increases macrophages and activated microglia cells that result in a larger lesion volume (Klusman and Schwab, 1997). Inhibiting TNF-α after injury using antibodies or anti-inflammatory cytokines improves neurological outcomes (Bethea et al., 1999; Lavine et al., 1998). These studies suggest that TNF-α is neurotoxic; however, TNF-α, at later stages of secondary injury is beneficial (Pan et al., 2002; Yan et al., 2001). Applying TNF-α 4 days after injury reduces lesion size (Klusman and Schwab, 1997). TNF-α receptor knock out mice result in diminished functional outcomes and longer lesions (Kim et al., 2001). TNF-α is neuroprotective against increased vascular permeability, neuronal and oligodendrocyte death (Donnelly and Popovich, 2008).

In contrast, IL-10 is a powerful anti-inflammatory cytokine (Brewer et al., 1999). IL-10 is also produced by microglial, macrophages and astrocytes (Geng et al., 1994; Mizuno et al., 1994).
IL-10 receptor proteins have been located on neurons, microglia, astrocytes and oligodendrocytes (Frei et al., 1994; Jander et al., 1998). Applying IL-10 reduces TNF-α synthesis in the spinal cord after injury, improves functional recovery and reduces lesion volume (Bethea et al., 1999). IL-10 knockout mice display reduced functional recovery and enhanced apoptosis, tissue damage, neutrophil infiltration, TNF-α and IL-1β expression after SCI (Genovese et al., 2009). Additional research is needed to elucidate the full scope of inflammation in SCI.

1.2.4 Cell death

After SCI, cell death occurs through two processes, necrosis and apoptosis. Necrotic death originates from the mechanical damage, excitotoxicity and oxidative stress sustained by the cells and characterized by cell swelling, organelle (mitochondria) damage, membrane lysis and release of intracellular contents (Kwon et al., 2004). Damaged mitochondria lead to ATP loss. High levels of intracellular Ca2+ activate enzymes such as phospholipase, proteases (see above) and endonucleases break down lipids and proteins resulting in membrane lysis and rupture that can start an inflammatory reaction (Hausmann, 2003; Kwon et al., 2004).

Apoptosis is characterized by cell and nuclear shrinkage with organelles intact and fragmentation into apoptotic bodies (Beattie et al., 2000; Crowe et al., 1997; Lu et al., 2000; Raff, 1998). In contrast to necrosis, apoptosis requires active participation of the cell and ATP. Apoptosis is initiated by ischemia, oxidative stress, excitotoxicity and TNF-α and occurs in all cell population of the spinal cord (Kwon et al., 2004; Yong et al., 1998). Apoptosis of oligodendrocytes has been demonstrated in rats, monkeys and humans after SCI (Crowe et al., 1997; Li et al., 1996). About 70% of apoptosis is of microglial/macrophage origin (OX42 positive) and around 15% profiles are from oligodendrocytes (CC1 positive) (Stirling et al., 2004). Apoptosis is mediated by the activation of protease enzyme, cysteine-dependent aspartate-specific protease (Caspase). Caspase-3, 5 and 9 are both elevated at 4 hours and 1 day after injury (Emery et al., 1998; Huang et al., 2000; McEwen and Springer, 2005; Springer et al., 1999). Caspase-3, the executioner of apoptosis, is activated by two pathways: intrinsic and extrinsic. Damaged mitochondria can release cytochrome c (Kluck et al., 1997) and trigger the intrinsic pathway by binding to apoptotic protease activating factor-1 (APAF-1). APAF-1 recruits pro-caspase-9 in the cytosol that resides in the mitochondria intermembrane space before apoptosis is triggered.
Once procaspase-9 is activated by proteolysis, Caspase-9, the initiator of apoptosis, becomes active. Caspase-9 initiates Caspase-3 that mediates the morphological and biochemical properties of apoptosis mentioned above. The extrinsic pathway is mediated by cytokine, TNF-α. TNF-α binds to TNF receptor on the membrane and death inducing receptor complex can be formed. Procaspsase-8 or 10 binds to the receptor complex by auto-activation. The activated Caspase-8 or 10 in turn, activates Caspase-3.

In contrast to necrosis that happens within hours after injury, the delayed nature of apoptosis makes it possible that interventions might halt the cell death process. Reducing this process will spare more tissue that can result in better functional outcome after spinal cord injury.
1.3 Experimental Injury Models

In order to study SCI, it is important to have an injury model that is well controlled, reproducible and an close representation of the clinical conditions (Anderson and Stokes, 1992). An effective experimental model must produce consistent injuries with little variability and that result in repeatable anatomical and functional outcomes (Behrmann et al., 1992; Behrmann et al., 1994; Black et al., 1986; Bresnahan et al., 1987; Bresnahan et al., 1991; Gruner, 1992; Panjabi and Wrathall, 1988; Wrathall et al., 1985). Efforts to develop an injury model started in 1911, when Reginald Allen introduced a standardized experimental weight-drop technique (Allen, 1911). In this system, gravity is used to produce various forces by releasing different weights from various heights. The modified and improved versions of gravimetric injury models are still in use. The New York University (NYU) impactor is a more sophisticated version that uses electromechanical components to measure injury displacement and velocity during weight drop (Basso et al., 1996; Gruner, 1992). On the other hand, the Ohio State University (OSU) impactor and the multimechanism injury system designed by Choo et al. (2009) physically sets the maximum displacement of the impactor tip to produce contusive SCI (Jakeman et al., 2000; Stokes, 1992). While these injury devices offer various advantages to generate injuries, none of these impactors directly controls the force delivered to the spinal cord, rather the force that applied to the cord is estimated. These theoretical forces do not always represent the actual energy that was transferred to the spinal cord, so it would be useful to measure the applied force during the injury to produce a precise and reproducible injury.

1.3.1 Cervical Contusion Models

Most SCI are incomplete and occur at the cervical level (NSCISC, 2009). In spite of the higher prevalence of cervical injuries, most SCI preclinical research is conducted in thoracic level. Among the various cervical injury models, contusion and compression injuries closely imitate the pathophysiological process observed in human SCI (Gensel et al., 2006; Kwon et al., 2002; Kwon et al., 2010a). For this reason, most neuroprotective therapies are examined in contusion models of SCI. This is not surprising as more than 75% of the SCI is caused by some type of blunt traumatic insult (Christopher and Dana Reeve foundation, 2009). In addition, most clinicians and scientists
agree that contusion injury is the most clinically relevant injury model of SCI (Kwon et al., 2010a). In preclinical SCI research, there are bilateral/midline and unilateral contusion models. Table 1.1 and 1.2 contains a comprehensive systematic review of cervical unilateral and bilateral/midline contusions.

In these models of SCI, the energy is absorbed by both dura and spinal cord. In contusion injuries, there are three biomechanical parameters that are important to injuring a spinal cord: load, displacement, and velocity (Briewener; Sparrey et al., 2008). Load is the force that is applied to the dura and in turn energy transferred to spinal cord. Displacement is the distance spinal cord is distorted by the force that was applied. Sparrey et al. (2008) demonstrated that with faster velocity there was a marked increase in hemorrhage volume and axonal disruption. In terms of injury mechanics, the three parameters are tightly linked together, so changing any one of these parameters has a significant impact on the extent of damage incurred by the spinal cord. Generally, the higher the velocity, force or displacement, the more severe the spinal cord is injured; however, setting two of these variables constant is crucial to produce controlled, reliable and reproducible injuries.

1.3.2 Infinite Horizon Spinal Cord Injury Device

The Infinite Horizon Spinal Cord (IH) impactor is a commercially available contusion device that enables an experimenter to set the force. The impactor takes advantage of the availability of extremely fast and programmable microcontrollers in order to sense force during impact. It is user friendly, so it does not involve complicated calibration steps before use. A range of forces can be set and once triggered, the impactor continues to displace forward (and thus deform the spinal cord tissue) until the desired force is reached, after which the tip is withdrawn. Force, velocity and displacement are immediately displayed in both a graphic and numeric output allowing the experimenter to evaluate the quality of the injury as the experiment progresses. The IH impactor is widely used in the thoracic injury model, as it has been shown that it produces consistent and reproducible contusion injuries (Iannotti et al., 2004; Radojicic et al., 2007; Scheff et al., 2003; Yu et al., 2008). Further discussion of this injury model is included in Chapter 2.
1.4 Neuroprotective Therapies

Many strategies are under extensive research in an attempt to treat SCI. As primary injury is beyond therapeutic management, the delayed nature of secondary provides a window of opportunity to reduce the pathophysiological process before the injury is aggravated. Neuroprotection is defined as measures to counteract secondary injury mechanisms and/or limit the extent of damage caused by self-destructive cellular and tissue processes (Anderberg et al., 2007).

Considering the overall complexity of secondary injury, it would be highly unlikely that implementing and targeting one mechanism will completely reverse secondary damage after SCI. Due to its safety profile and clinical applicability, one area that is under active investigation is the evaluation of pharmacological agents that are already in clinical use, albeit for different indications, that may target multiple secondary mechanisms. Especially for cervical injuries, this strategy will have a dramatic impact to patients, since even a little preservation in the cervical level can potentially lead to significant functional differences and improved quality of life (van Hedel and Curt, 2006).

Minocycline and simvastatin are two drugs that fit these criteria. They are already in clinical use and have been shown to target multiple secondary mechanisms in various neurological diseases.

1.4.1 Minocycline

Minocycline, a tetracycline derivative, has been traditionally used clinically to treat inflammation related conditions such as acne or rheumatoid arthritis due to its antimicrobial properties. Minocycline is highly lipophilic so it readily crosses the BBB (Klein and Cunha, 1995) and the safety profile of minocycline has been well established. For these reasons, minocycline has been extensively investigated to elucidate the therapeutic mechanisms in many clinical indications such as TBI (ClinicalTrials.gov Identifier: NCT01058395), stroke (ClinicalTrials.gov Identifier: NCT00930020, NCT00836355), ischemia (ClinicalTrials.gov Identifier: NCT00630396), multiple sclerosis (ClinicalTrials.gov Identifier: NCT01134627), Parkinson’s disease (ClinicalTrials.gov Identifier: NCT00063193) and Huntington’s disease (ClinicalTrials.gov Identifier: NCT00029874). It was discovered that minocycline inhibits apoptosis, inflammation, oxidative stress and
excitotoxicity in the CNS. In SCI, investigations in minocycline have been well illustrated in a recently published table by Kwon et al. (Kwon et al., 2010b)(Table 1.3). In summary, Yrjanheikki et al. (1999) and Tikka et al. (2001) demonstrated that minocycline reduced microglia activation and alleviated excitotoxicity (NMDA receptor) mediated neuronal death in spinal cord culture. The first in vivo study was reported by Wells et al. (2003) where minocycline (50mg/kg, 1 hour post and 25 mg/kg/day for 5 days, IP) in a mice thoracic clip compression model promoted an early functional improvement on the hind limb motor score and reduction of lesion size. In the same year, Lee et al. (2003) corroborated that minocycline (90mg/kg/day for 38 days, IP) reduced lesion size and improved functional outcomes in a rat thoracic contusion injury model. Furthermore, they demonstrated that the mechanism behind minocycline’s therapeutic effects was correlated to reducing apoptosis 24 hours post-injury, inhibiting Caspase-3 activity at 4 hours, decreasing TNF-α expression and increasing anti-inflammatory cytokine, IL-10 6 hours after SCI. Stirling et al. (2004) further investigated the cellular and molecular basis of minocycline (90 mg/kg/day for 3 days, IP) mediated neuroprotection and reported that with minocycline treatment, there were a marked reduction in apoptosis of oligodendrocytes, microglia and macrophages that led to less axonal dieback. Teng et al. (2004) demonstrated that minocycline treatment (90 mg/kg/day, IP for 5 days) reduced cytochrome c release from the mitochondria, reduced astrogliosis and preserved more oligodendroytes. Festoff et al. (2006) demonstrated that the therapeutic effects of minocycline is similar for multiple administration with reduced dose (30mg/kg, IP at 30 mins, 1 hour and 24 hours after injury) compared to one bolus administration (90mg/kg, IP at either 30 mins, 1 hour or 24 hours after injury) as long as it was administered within 24 hours after injury. This study confirmed the reduction of Caspase-3 activity and apoptosis with minocycline administration. Yune et al. (2007) showed that minocycline (90 mg/kg/day, IP for 4 days), inhibited pro-nerve growth factor (NGF) expression and reduced oligodendrocyte death after injury. They have further identified that it was microglia that was secreting proNGF by phosphorylating p38 MAPK downstream pathway. Inhibiting oligodendrocyte apoptosis also improved functional recovery. These beneficial effects of minocycline are mediated by targeting multiple secondary injury mechanisms and reduce further exacerbation of injury.

However, more recently, minocycline’s therapeutic effects have been questioned. In a NIH funded replication study by Pinzon et al. (2008) the therapeutic effects reported by Lee et al. were not reproduced. In a balloon compression model, Saganova et al. (2008) reported limited
neurological improvements after minocycline. In addition, we reported that in a thoracic contusion injury using the OSU impactor and in an unilateral cervical contusion injury using the Infinite Horizon impactor (Chapter 3), minocycline did not display functional and histological improvements after SCI which supports the findings of Pinzon et al. (Mann et al., 2010; Lee et al., 2010). Minocycline is currently in phase two clinical trial. The findings so far indicate that the therapeutic effect of minocycline is selective to cervical incomplete SCI patients (ClinicalTrials.gov Identifier: NCT00559494).

1.4.2 Simvastatin

Statins were initially discovered to reduce cholesterol synthesis by inhibiting mevalonate production. Cholesterol is synthesized when Acetyl-coenzyme-A becomes squalene through multiple steps (Figure 1.1). Acetyl-coenzyme-A is converted to acetoacetyl-coenzyme-A and in turn into hydroxymethylglutaryl-coenzyme-A (HMG-CoA) by HMG-CoA synthase. HMG-CoA reductase produces mevalonic acid from HMG-CoA. Mevalonic acid becomes mevalonate by mevalonate kinase. Mevalonate becomes mevalonate pyrophosphate, isopentyl pyrophosphate (PP), geranyl PP and farnesyl PP by phosphomevalonate kinase, mevalonate PP decarboxylase, geranyl PP synthase and farnesyl PP synthase, respectively. When squalene synthase converts farnesyl PP to squalene, cholesterol is synthesized (Buhaescu and Izzedine, 2007). When farnesyl PP is converted to geranylgeranyl PP by geranylgeranyl PP synthase, both farnesyl PP and geranylgeranyl PP attaches to various proteins, such as G proteins and Ras, Rho, Rap and Rab GTPases, GTP-binding proteins for post-translational modification: a process referred to as isoprenylation (Hall et al., 1998; van der Most et al., 2009; Van and Souza-Schorey, 1997)(Figure 1.1). Isoprenylation is important in protein-protein interactions to properly target proteins to specific locations (Zhang and Casey, 1996). The rate limiting enzyme in this process is HMG-CoA reductase and statins can reduce cholesterol production by inhibiting HMG-CoA reductase (Rikitake and Liao, 2005). The inactivation of HMG-CoA reductase by statins can target multiple secondary injury mechanisms making it an attractive neuroprotective therapy. For example, statins can inhibit inflammation, oxidative stress and RhoA to promote axonal regeneration.

The Rho pathway is known for its role in influencing axon elongation and growth cone turning (Borisoff et al., 2003; Chan et al., 2005). After SCI, inhibitory factors such as, Nogo-A
protein, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) and their receptor Nogo-66 (NgR) and p75NTR (Chen et al., 2000; Fawcett and Asher, 1999; Fitch and Silver, 1997; GrandPre et al., 2000; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Niederost et al., 1999; Prinjha et al., 2000; Shearer and Fawcett, 2001; Stichel and Muller, 1998; Wang et al., 2002) and the scar tissue rich in CSPG can activate RhoA/Rho-kinase pathway (Madura et al., 2004; Nishio et al., 2006). Rho pathway activation leads to growth cone collapse and apoptosis of neurons and oligodendrocytes after injury inhibiting axonal regeneration (Dubreuil et al., 2003). GTP-bound RhoA level increases and peaks at 5 days after SCI. Inhibiting Rho pathway has been shown to partially promote axonal sprouting and regeneration after SCI and protects cells from apoptosis (Dubreuil et al., 2003).

Statins like minocycline have also been reported to show therapeutic effects in various neurological diseases such as TBI, MS, stroke. The up to date comprehensive review of statins in SCI is in Table 1.4. In SCI, Pannu et al. (2005) was the first to report that in vivo, atorvastatin (Lipitor®) dramatically increased functional recovery and histological outcomes in a rodent contusion model. In addition, atorvastatin has been shown to reduce RhoA activity, neutrophil and macrophage infiltration and MMP-9 expression (Pannu et al., 2007). Recently, the neuroprotective effect of atorvastatin has been confirmed by Dery et al. (2009). Simvastatin is one of the most potent statins (Schachter, 2005). It is more lipophilic compared to atorvastatin so it readily crosses the BBB. These properties make simvastatin a better theoretical drug; however, there are conflicting reports regarding the neuroprotective effects of simvastatin. In SCI, the beneficial effects of simvastatin (Zocor®) was first reported in vitro by Holmberg et al. (2006), where simvastatin promoted neurite outgrowth in the presence of growth inhibitory molecules such as MAG, OMgp and Nogo. Subsequently, simvastatin has been shown to decrease CSPG intensity (Holmberg et al., 2008). To date, there is only one study that reported functional recovery after SCI. Oral administration of simvastatin was reported to improve the Basso, Beattie and Bresnahan locomotor score after contusive spinal cord injury (Shunmugavel et al., 2010). On the other hand, Holmberg et al. (2008) failed to demonstrate functional improvements and the lack of neurological benefits of atorvastatin and simvastatin have been reported in a thoracic contusion model (Mann et al., 2010).
1.5 Objectives

The first step towards evaluating potential treatments is to establish an injury model that accurately represents the clinical condition. Currently, the majority of SCI research is conducted at the thoracic level. There are hundreds of therapies that are under preclinical investigation as a potential treatment for SCI (Kwon et al., 2010b; Kwon et al., 2010c; Tetzlaff et al., 2010). Some of these have been tested on humans, only to reveal they are unable to reproduce the results seen in experimental studies (Tator, 2006). I have chosen minocycline and simvastatin as these agents are considered to be “safe to use” in patients with SCI and have demonstrated the potential to improve neurological outcomes. My objectives are to:

1. Develop a clinically relevant cervical unilateral contusion model using the Infinite Horizon Spinal Cord Injury device.

2. Determine if minocycline or simvastatin improve neurological recovery in this cervical injury model.

The objectives will be addressed in chapters 2, and 3. The experimental model of spinal cord injury that we have chosen to develop is the cervical unilateral contusion using the IH impactor that consistently delivers a blunt force to the spinal cord using a force feedback-controlled mechanism. The contusive nature of injury model is thought to best stimulate the biomechanics and pathology of human spinal cord injury (Kwon et al., 2010a). This injury model was developed using a host of behavioral tests such as Horizontal ladder test, Cylinder rearing test, modified Montoya staircase test and grooming test. Then, the neuroprotective properties of minocycline and simvastatin were evaluated using this cervical unilateral contusion model. The rationale for testing these drugs will be further expanded and the specific hypotheses of my experiments will be outlined in the introduction of each chapter.
Table 1.1 Unilateral Cervical Contusion Models in Spinal Cord Injury.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Animal model</th>
<th>Injury parameters</th>
<th>Reported Outcome</th>
<th>Note</th>
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</thead>
</table>
| Lee et al. Chapter 2,3 of this thesis | Model: male SD, 300 – 350g, C5 unilateral contusion                       | IH, 150 kdyne, displacement: 1.48–1.55 mm | **Functional outcome:** @ 6 weeks  
-Horizontal Ladder test: ipsilateral percent error = ~20-25%  
-Cylinder rearing test: ipsilateral percent usage = ~20%  
-Grooming test = ~2  
-Modified Montoya staircase test = ~5-7 pellets  
**Histological Outcome:**  
-Epicenter spared white matter = ~20%, epicentre spared grey matter = ~20%  
-Cumulative white matter = ~50%, cumulative grey matter = ~50% |        |
| Lee et al. Spine, 2010 (in press) | Model: male SD, 300 - 350g, C4/5 unilateral contusion                      | OSU, 1.5 mm displacement: 100-200 kdyne | **Functional outcome:** @ 6 weeks  
-Horizontal ladder test: ipsilateral percent error = ~11.02%  
-Cylinder rearing test: ipsilateral percent usage = ~28.0%  
-Catwalk: %Ab = 25% decrease (@62%), L/R ratio print area = ~30% decrease, L/R swing duration = ~60% increase, L/R ratio swing speed = ~30% decrease, Sensory test: ipsilateral withdrawal force: 35.0g, latency: 4.8s  
**Histological Outcome:**  
-Epicenter spared white matter = ~40%, epicenter spared grey matter = ~10%  
-Cumulative white matter = ~50%, cumulative grey matter = ~30% |        |
| Sandrow et al., J. Exp. Neurol., 2008, 2010/ J. Neurotrauma, 2009 | Model: female SD, 225 – 250g, C4 or C5 unilateral contusion. | IH, 200 kdyne, displacement: 1.6–1.8 mm, | **Functional outcome:** @ 8 weeks  
-Forced locomotion (Tread Scan) print area: 600-750 pixels,  
-FLAS: 12-13,  
-BBB scale (right hindlimb):  
-Grid walk test %correct placement: ~80–~90%,  
-Grip strength test: 85% baseline.  
**Histological Outcome:**  
-Percent total spared tissue = ~50%  
-Epicenter spared tissue: white matter = ~45%, grey matter = ~20%  
-Longitudinal lesion extent: 130 mm. | 1.6 mm impactor tip immersed in water. |
| Baussart et al., Neurobiology of Disease, 2006 | Model: female SD, 225 – 275g, C2 unilateral contusion. | Weight drop, diameter: 0.8mm, length: 120 mm, weight: 20 g, height: 12 mm, | **Functional outcome:** Not reported  
**Histological Outcome:** Not reported | Dura and pia removed, injury for unilateral diaphragm deficits. |
<p>| Gensel et al., J. | Model: female Long- NYU, weight: 10 g, | | <strong>Functional outcome:</strong> @ 6 weeks. | Injury crossover. |</p>
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<tr>
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<th>Injury parameters</th>
<th>Reported Outcome</th>
<th>Note</th>
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</table>
| Neurotrauma, 2006 | Evans, 10 – 12 weeks (average 217g), C5 unilateral contusion. | height: 6.25 mm, 12.5 mm, tip diameter: 2.0 mm, | -Grooming test: 6.25mm=4.3, 12.5mm=3.4.  
-Cylinder rearing test (contralateral usage): 6.25mm=55%, 12.5mm=98.3%.  
-Horizontal ladder test (% correct placement): 6.25mm=95%, 12.5mm=85%, (% rungs used): no change  
-Automated walkway (time to cross, distribution of total steps, stride length): 6.25, 12.5mm= no change, (print area reduction): 6.25mm=48.1%(41 mm²), 12.5mm=67.1%(26 mm²).  
**Histological Outcome:**  
-White and grey matter area: severity dependent tissue loss  
lesion epicenter area: %white matter: 6.25mm=10%, 12.5mm=5%, %grey matter: 6.25mm=50%, 12.5mm=20%  
-3D reconstruction,  
-Myelin density (rostral): 6.25mm=~80.0%, 12.5mm=~73.3%,  
(caudal): 6.25mm=~76.6%, 12.5mm=~66.7%  
-Motor neuron count (r0.6-c0.6mm): 6.25, 12.5mm=~0, (@r1.0,c1.0mm): 6.25, 12.5mm=~5,  
(@r1.4,c1.4mm): 6.25, 12.5mm=~10. | |
| Soblosky et al., Behav. Brain Research, 2001 | **Model:** female SD, 240 - 270g, C4/5 unilateral contusion. | Weight drop (Allen’s), weight: 10.5 g, height: 1.25, 2.50, or 5.00 cm, tip diameter: 1.6 mm, | **Functional outcome:** @ 12 weeks  
-Horizontal Ladder test, misplacements: 1.25cm=2.5, 2.50cm=6, 5.00cm=6.5, slips: 1.25cm=0.25, 2.50cm=0.75, 5.00cm=1.75,  
-Cylinder Rearing test, percent (uninjured) usage: 1.25cm=65%, 2.50cm=75%, 5.00cm=90%,  
**Histological Outcome:**  
-Spared white matter: 1.25cm=1.21 mm², 2.50cm=0.71 mm²,  
5.00cm=0.33 mm²,  
-Spared grey matter: 1.25cm=1.00 mm², 2.50cm=0.61 mm²,  
5.00cm=0.30 mm². | Impact at 20 degree angle. |

SD: Sprague Dawley; R: Right; L: Left; IH: Infinite Horizon Spinal Cord Injury Impactor; OSU: Ohio State University Impactor; NYU: New York University Impactor; C: cervical vertebra; r: rostral; c: caudal; BBB: Basso, Beattie and Bresnahan locomotor test.
## Table 1.2 Bilateral/Midline Cervical Contusion Models in Spinal Cord Injury.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Animal model</th>
<th>Injury parameters</th>
<th>Reported Outcome &amp; Note</th>
<th>Note</th>
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| Sharp et al., Stem Cells, 2010 | Model: female SD, 200 – 220g | IH, 200 kdyne, displacement: not reported, C5 bilateral contusion | **Functional outcome:** @ 9 weeks  
- Footprint analysis: stride length=68.6%(12 cm), proximal forelimb motion range=70%, proximal forelimb angle(lift off)=90%, (placement)=20%, percent forelimb steps passed 90° =40%.  
**Histological Outcome:**  
- Lesion area: 6.5mm²,  
- Spared white matter: 1.0mm², spared grey matter: 0.1mm²,  
- Spared ventral grey matter: 0.1mm²,  
- Average spared motor neurons: 5. | |
| Anderson et al., J. Exp. Neurol., 2009 | Model: female SD, 200 – 230g | IH, 200 and 250 kdyne, C5, C6, or C7/8 bilateral contusion | **Functional outcome:** @ 8 weeks  
- Forelimb locomotor assessment scale (FLAS): 200kdyne=50, 250kdyne=40.  
- Grip strength test, C5RL.C6L:200kdyne=~25g, C6R:200kdyne=~0.0g, C6RL:250kdyne=~0.0g, C7/8L:200kdyne=~25.0g, C7/8R:200kdyne=~50.0g, C7/8RL:250kdyne=~15.0g.  
- Sensory testing: 200kdyne=0.15, 250kdyne=0.15.  
**Histological Outcome:**  
- CST axon counting (caudal): dorsal CST: 200kdyne=0.0, 250kdyne=0.0, dorsal lateral CST: 200kdyne=59, 250kdyne=28.8, ventral CST: 200kdyne=5.9, 250kdyne=2. | 3.5 mm impactor tip |
| Choo et al., J. Neurosci. Methods, 2009 | Model: male SD, 295 - 337g | multi-mechanism injury system, displacement: 1.1 mm, C4/5 bilateral contusion. | **Functional outcome:** Not reported  
**Histological Outcome:**  
- Hemorrhage analysis (volume): 0.82 mm³,  
- White matter pathology: damage concentrated centrally  
- Nodes of Ranvier length increase: lesion epicentre =9.52%(11.5µm). | Custom designed impactor |
| Onifer et al., J. Exp. Neurol., 2007 | Model: male SD, 315 – 443g. | IH, 176 – 201 kdyne, C5/6 bilateral contusion. | **Functional outcome:** Not reported  
**Histological Outcome:**  
- Dorsal column area: 73.4% (1.02mm²). | Injury to study forelimb evoked potential. |
| Collazos-Castro et al., J. Neurosurg. Spine, 2005 | Model: male Wistar, 7 – 8 weeks old. | weight drop (Allen), weight: 10 g, height: 12.5 mm, C7 bilateral contusion. | **Functional outcome:** @ 12 weeks  
- Kinematics: reduced elbow extension =18.1%(118°), increased stance phase =76.9%(0.46s), decreased mean walking velocity =48.6%(0.18m/s).  
**Histological Outcome:**  
- Dorsal corticospinal tract tracing, longitudinal lesion extent. | Rectangle impactor. |
<table>
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<th>Injury parameters</th>
<th>Reported Outcome</th>
<th>Note</th>
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</table>
| Pearse et al., J. Neurotrauma, 2005 | Model: female Fischer, 180 – 200g.| OSU, tip diameter: 4 mm, displacement: 0.8, 0.95 or 1.10mm, C5 bilateral contusion. | **Functional outcome**: @ 8 weeks,  
-Cylinder rearing test single forelimb usage increase: 0.8mm=no change (75.9%), 0.95mm=16.8%(91.0%), 1.10mm=unable to rear.  
Both forelimb usage reduction: 0.8mm=no change(24.1%), 0.95mm=61.5%(9.0%), 1.10mm=unable to rear.  
-BBB scale: 0.8mm=15, 0.95mm=10.3, 1.10mm= 8.3.  
-Weight-support forelimb hanging test time:0.8mm=7.8s(72.9%), 0.95mm=4.7s(43.9%), 1.10mm=1.2s(11.2%).  
-Grip strength test: 0.8mm=0.85Nm (79.3%), 0.95mm=0.58Nm(54.2%), 1.10mm=0.11Nm(10.3%).  
-Bidirectional inclined plane (head up): 0.8mm=57.7°(100%), 0.95mm=52.0°(81.3%), 1.10mm=48.4°(84.0%). (head down): 0.8mm=55.4°(100%), 0.95mm=50.1°(80.1%), 1.10mm=38.8°(69.8%).  
-Grid walk test (Buhaescu and Izzedine, 2007): 0.8mm=2.1, 0.95mm=11.2, 1.10mm=13.3.  
-Contact-placement test (unsuccessful attempts): 0.8mm=15.2%, (no change), 0.95mm=57.9%, 1.10mm=98.5%  
-White matter volume: 0.8mm=66.3%, 0.95mm=43.1%, 1.10mm=18.5%.  
-Grey matter volume: 0.8mm=73.1%, 0.95mm=49.0%, 1.10mm=0.5%.  
-Severely dependent myelinated axon loss,  
-Neuronal cell preservation (percent loss) (Dorsal horn): 0.8mm=r600(45.6%)-c600µm(47.3%), 0.95mm=r900(37.8%)-c1200µm(35.1%), 1.10mm=r1200(64.9%)-c1500µm(34.6%), epicentre: 0.8mm=82.1%(67.6), 0.95mm=94.9%(19.4), 1.10mm=99.8%(0.6).  
-Longitudinal lesion extent: 3.06mm. |                          |
| Collazos-Castro et al., J. Neurotrauma, 2005 | Model: male Wistar, 400 – 450g (18 -24 weeks old). | Weight drop (Allen’s), weight: 10 g, height: 12.5 mm, C7 bilateral contusion. | **Functional outcome**: @ 25 weeks  
-3D kinematics: increased stance phase duration=66.7%(0.5s), increased walking cycle duration =x2(0.8s), decreased mean walking velocity =x2(0.15m/s).  
**Histological Outcome**:  
-Motor neuron count: HRP labelled triceps motoneurons: 54%(Kluck et al., 1997)  
-Dorsal corticospinal tract tracing: aminostibamidine labelled motoneurons: 63%(159.6)  
-Longitudinal lesion extent: 3.06mm. | **Rectangle impactor.**                                      |
| El-Bohy et al., J. Exp. Neurol., 1998 | Model: female SD, 200 – 275g | NYU, height: 12.5 mm, C2 lateral, C4/5 bilateral contusion | **Functional outcome**: Not reported  
**Histological Outcome**: Not reported | Injury developed for respiratory function assessment. |

**SD**: Sprague Dawley; **R**: Right; **L**: Left; **IH**: Infinite Horizon Spinal Cord Injury Impactor; **OSU**: Ohio State University Impactor; **NYU**: New York University Impactor; **C**: cervical vertebra; **r**: rostral; **c**: caudal; **BBB**: Basso, Beattie and Bresnahan locomotor test.
Table 1.3 The Evaluation of Minocycline in Spinal Cord Injury (Kwon et al., 2010)

<table>
<thead>
<tr>
<th>Paper</th>
<th>Animal Model and Injury Model</th>
<th>Intervention and Timing</th>
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<th>Reported Outcomes: • Histologic / Biochemical / Physiological • Behavioral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee Chapter 3 of this thesis</td>
<td>Model: Adult Male SD Rats, 300-350g Injury: C5 Left Hemicontusion</td>
<td>Minocycline IP • 90 mg/kg @ 1hr PI, then 45 mg/kg IP at 12h and 24h</td>
<td>SCI+ - Minocycline (n=9) - Saline IP (n=10)</td>
<td>Behavioral: Minocycline did not lead to behavior improvement at any time point (horizontal ladder test, cylinder rearing test, modified Montoya staircase test, grooming test and sensory test). Histologic / Biochemical / Physiological: Minocycline did not improve spared tissue areas calculated from the cross sections.</td>
</tr>
<tr>
<td>Marchand Eur J Pain 2008</td>
<td>Model: Adult Male Wistar Rats, 220–250g Injury: T13 Left Hemisection</td>
<td>Minocycline IP • 40 mg/kg @ 30 min PI, then 3 times q12h.</td>
<td>SCI+ - Minocycline (n=8) - Saline IP (n=8)</td>
<td>Behavioral: Minocycline treatment prevented the development of mechanical allodynia and thermal hyperalgesia in both ipsilateral and contralateral paws during first 2 w PI. Histologic / Biochemical / Physiological: Minocycline significantly attenuated microglial activation by 30-50% in the lumbar dorsal horns (OX-42 expression, n=4) and evoked neuronal activity (c-Fos expression after noxious stimulation, n=4) at 7d and 14d PI.</td>
</tr>
<tr>
<td>Ha Eur Spine J 2008</td>
<td>Model: Male SD Rats, 300–350 g Injury: T9/10 NYU Impactor 10g x 25 mm</td>
<td>Minocycline IP or IV • 30 mg/kg @ 30min PI, then 12h, 24h, 36h and 48h PI Methylprednisolone IP • 30 mg/kg IP, @ 30 min, then at 12h and 24 h PI</td>
<td>SCI+ - Minocycline (n=8) - MP (n=8) - Saline IV (n=8)</td>
<td>Behavioral: Minocycline significantly improved locomotor function at 7d PI (2.6 ± 0.5 vs. 1.0 for saline and 2.4 ± 0.5 for MP group in 6-point locomotion scale) and also improved incline plane test score at 7d PI. Histologic / Biochemical / Physiological: Minocycline decreased lesion volume, attenuated microglial activation (anti-OX-42+ cells) and apoptosis (TUNEL).</td>
</tr>
<tr>
<td>Pinzon Brain Res 2008</td>
<td>Model: Male SD Rats, 220–280g Injury: T9/10 NYU Impactor 10g x 12.5 mm</td>
<td>Minocycline IP or IV • 90 mg/kg @ 0h PI, then 45 mg/kg IP at 12h and 24h PI</td>
<td>SCI+ - Minocycline IP (n=15) - Minocycline IV (n=15) - Vehicle IP (n=8) - Vehicle IV (n=7)</td>
<td>Behavioral: Minocycline did not lead to behavioral improvement at any point in time (BBB: minocycline IP 12.1±0.18 vs saline control 11.8 ±0.2; differences in BBB subscores were also not observed). Histologic / Biochemical / Physiological: Minocycline did not improve spared tissue areas or total cavity areas as calculated from horizontal sections.</td>
</tr>
<tr>
<td>Saganova Neurosci Lett 2008</td>
<td>Model: Adult Wistar Rats 300-330g Injury: T9 Balloon Compression (12.5µl x 5 min)</td>
<td>Minocycline IP • 90 mg/kg @ 1h PI, then 45 mg/kg IP at 12h and 24h • 90 mg/kg @ 1h PI, then 45 mg/kg IP q12h x 5 days</td>
<td>SCI+ - Minocycline x 1 day - Minocycline x 5 days - Saline IP (n=12/group)</td>
<td>Behavioral: Minocycline at both the 1 and 5 day administration regimen did not result in any BBB improvement over 28 days of observation. Histologic / Biochemical / Physiological: Minocycline at both the 1 and 5 day administration regimen increased gray and white matter sparing in sections 2-4 mm rostral to injury epicenter, but did not influence tissue sparing at the epicenter or at any sections caudal to epicenter.</td>
</tr>
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<td>Yune</td>
<td>Model: Male SD Rats, 250-300g</td>
<td>Minocycline IP • 90 mg/kg @ 0h or 2h PI, then 45 mg/kg q12h for 3d. Methylprednisolone IP • 30 mg/kg @ 0h or 2h PI, then 30 mg/kg q12h for 3d.</td>
<td>SCI + - Minocycline (n=44) - MP (n=20) - Vehicle (n=44) Sham (n=3)</td>
<td><strong>Behavioral:</strong> Minocycline treatment (immediate or 2h delay) but not MP resulted in significantly improved BBB, inclined plane and grid walk stepping. Delayed (2 h) minocycline also improved foot coordination (foot print analysis), whereas MP did not. <strong>Histologic / Biochemical / Physiological:</strong> Minocycline significantly reduced levels of pro-NGF by 5 d post-SCI, protein levels of p-p38MAPK and p-MAPKAPK-2 by 3d and 5d post-SCI, significantly inhibited p75NTR mRNA and protein, and GTP-bound RhoA at 3d and 5d, significantly decreased the number of caspase-3-positive (CC1-positive) oligodendrocytes, all while sparing myelin and reducing axonal loss.</td>
</tr>
<tr>
<td>Festoff</td>
<td>Model: Female SD Rats, 300-325g</td>
<td>Minocycline IP • Single dose 90 mg/kg @ 30min, 1h or 24h PI. • 3 doses of 30 mg/kg @ 30min, 1h and 24 h PI. Tetracycline IP • 30 mg/kg @ 1 h PI.</td>
<td>SCI + - Minocycline (90 mg/kg @ 0.5,1 or 24 h post-SCI) (n=12) - Minocycline (30 mg/kg x 3) (n=4) - Tetracycline (n=3) Sham (n not indicated)</td>
<td><strong>Behavioral:</strong> Minocycline 30 mg/kg x 3 doses yielded ~5 point BBB improvement over tetracycline controls. Similarly, minocycline (single dose 90 mg/kg) given at 0.5, 1, or 24h post-SCI improved BBB scores over tetracycline-treated injured controls (Day 28: 14.6 ± 0.62 vs 8.33 ± 0.66). Timing of minocycline post-injury did not significantly affect functional recovery. <strong>Histologic /Biochemical / Physiological:</strong> Minocycline significantly spares tissue, reduces tissue damage, cavity size, gliosis, necrosis, apoptosis, caspase-3 activation and caspase-3 cleavage products. Minocycline also reduced microglial activation and TNF-α levels.</td>
</tr>
<tr>
<td>Stirling</td>
<td>Model: Adult Wistar Rats</td>
<td>Minocycline IP • 50 mg/kg @ 30 min PI and 8h PI, then q12 x 2d</td>
<td>SCI + - Minocycline - Saline n=6-8/group for 7d survival n=4-5/group for 14d survival</td>
<td><strong>Behavioral:</strong> Footprint analysis revealed improved interlimb coordination and reduced hindlimb angle of rotation with minocycline treatment. <strong>Histologic / Biochemical / Physiological:</strong> Minocycline greatly reduces active caspase-3-positive oligodendrocytes and microglia/macrophages profiles in proximal and distal ascending sensory tracts (AST), inhibited transection-induced glial cell death within the distal and proximal AST, reduced ED1-positive (microglial/macrophage) density 7d PI, and reduced corticospinal tract dieback and lesion size both 7d and 14d PI.</td>
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<td><strong>Teng</strong> PNAS 2004</td>
<td>Model: Female SD Rats, 280-330g Injury: T9 NYU Impactor 10g x 25 mm</td>
<td>Minocycline IP • 8, 90 or 180 mg/kg @ 1h PI, collected @ 4h PI (dose response study) • 90 mg/kg @ 1h PI, then 45 mg/kg q12h x 5d</td>
<td>SCI + • Minocycline • Vehicle n=3/group/dose and time point</td>
<td><strong>Behavioral:</strong> Minocycline-treated rats demonstrated significantly increased coordinated hindlimb motor function; 3w and 4w BBB scores were significantly greater for minocycline treatments vs. vehicle. <strong>Histologic / Biochemical / Physiological:</strong> A dose-dependent effect of minocycline on cytochrome c release at SCI site was seen at 4h PI, reducing cytochrome c release to the negligible pre-SCI level. At 28d PI, minocycline preserved residual white matter, protected ventral horn neurons and oligodendrocytes, and reduced reactive astroglia within the ventral funiculi.</td>
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<tr>
<td><strong>Lee</strong> J Neurotraum a 2003</td>
<td>Model: SD Rats, 230-250g Injury: T10 NYU Impactor 10g x 12.5 mm</td>
<td>Minocycline IP • 90 mg/kg @ 0h PI, then 45 mg/kg q12h x 2 doses</td>
<td>SCI + • Minocycline (n=41) • Vehicle (n=41) • Sham (n=13)</td>
<td><strong>Behavioral:</strong> Minocycline significantly increased BBB scores at 24-38 days PI (18.6±0.7 vs. 15.6 ±0.5). <strong>Histologic / Biochemical / Physiological:</strong> Minocycline reduced cavitation between 28 and 38 days, caspase-3 activity, TUNEL-positive cells, and DNA laddering.</td>
</tr>
<tr>
<td><strong>Wells</strong> Brain 2003</td>
<td>Model: Male CD-1 Mice ~3 months of age Injury: T3/T4 Clip Compression 8g</td>
<td>Minocycline IP • 50 mg/kg @ 1h PI, then 25 mg/kg q24h x 6d.</td>
<td>SCI + • Minocycline (n = 43) • Vehicle (n = 41)</td>
<td><strong>Behavioral:</strong> Minocycline improved murine survival, BBB and inclined plane. <strong>Histologic / Biochemical / Physiological:</strong> Minocycline treatment yielded increased rubrospinal tracts, and reduced lesion area.</td>
</tr>
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</table>

SCI: spinal cord injury; d: day, days; h: hour, hours; w: week, weeks; PI: post-injury; q24h: interval 24 hours; T: thoracic vertebra; C: cervical vertebra; IP: intraperitoneal; IV: intravenous; SC: subcutaneous; BBB: Basso, Beattie and Bresnahan locomotor test; MP: Methylprednisolone; SD: Sprague-Dawley; TNF: tumor necrosis factor
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<tr>
<td>Lee Chapter 3 of this thesis</td>
<td>Model: Adult Male SD Rats, 300-350g Injury: CS Left Hemicontusion</td>
<td>Minocycline IP  • 90 mg/kg @ 1h PI, then 45 mg/kg/day q12h x 2 doses for 3d  Simvastatin SC • 20mg/kg/day for 3d and 5mg/kg/day for 4d  Simvastatin SC • 20mg/kg/day for 3d and 5mg/kg/day for 39d</td>
<td>SCI +  • Vehicle IP, SC (n=10)  • Minocycline (n=9)  • Simvastatin 7days (n=10)  • Simvastatin 42days (n=10)</td>
<td>Behavioral: There were no significant differences between any of the groups in horizontal ladder test, cylinder rearing test, modified Montoya staircase test and grooming test. Histologic/Biochemical/Physiological: There were no significant improvements for Minocycline, Simvastatin 7 days and Simvastatin 42 days treated group in white and grey matter sparing and summed white and grey area between r400µm to c400µm.</td>
</tr>
<tr>
<td>Mann J. Exp. Neurol. 2010</td>
<td>Model: Adult Male SD Rats, 290-340g Injury: T9/10 contusion, 1.5 mm</td>
<td>Atorvastatin gavage @  • 5mg/kg/day  Simvastatin gavage @  • 20mg/kg/day  Simvastatin gavage @  • 20mg/kg/day 7d  Simvastatin SC • 20mg/kg/day for 3d and 5mg/kg/day for 4d</td>
<td>SCI +  • Atrovastatin (n=4: gavage, n=8:gavage, n=8: oral Ensure® )  • Simvastatin (n=4: gavage, n=8:gavage, n=8: oral Ensure®, n=16:20mg/kg/day and 5mg/kg/day)</td>
<td>Behavioral: There were no significant differences between Simvastatin and Atrovastatin treated groups in BBB score, horizontal ladder test and Catwalk. Histologic/Biochemical/Physiological: Proportion of cross section labeled by ED1 was significantly less in Simvastatin 20mg/kg/day compared to control. There were no significant improvements for Simvastatin and Atrovastatin treated group in white and grey matter sparing and summed white and grey area between r400µm to c400µm.</td>
</tr>
<tr>
<td>Ohsawa Pain 2008</td>
<td>Model: Male ICR mice, 20-30g</td>
<td>Simvastatin IP 10, 20 or 40 mg/kg/day</td>
<td>SCI -  • Simvastatin (n=10, 10mg/kg/day)  • Simvastatin (n=10, 20mg/kg/day)  • Simvastatin (n=10, 40mg/kg/day)  • Vehicle</td>
<td>Behavioral: Formalin induced nociceptive responds. First phase: no difference, Second phase: vehicle: 200s, 10mg/kg/day: 200s, 20mg/kg/day: 150s, 40mg/kg/day:100s. Simvastatin was used to deplete endogenous mevalonate.</td>
</tr>
<tr>
<td>Holmberg J. Exp. Neuro., 2008</td>
<td>Model: Female Long-Evens Rats, 200-220g Injury: T9 NYU Impactor 10g x 25 mm</td>
<td>Simvastatin(Sim) 0.57 or 2.3 mg/kg/day, gavage, 0.25 µl/h osmotic mini pump into the lateral ventricle</td>
<td>SCI +  • Simvastatin (brain pump)  • Simvastatin (low dose)  • Simvastatin (high dose)  • Vehicle  • Sham</td>
<td>Behavioral: There were no significant differences between any of the groups in the BBB score at 4 weeks. Histologic/Biochemical/Physiological: CSGP expression reduced ~66% in high dose Sim. Both high and low dose Sim in the nonlesion region reduced CSGP expression by ~66%. There were no changes in GFAP and MBP positive tissue area. Sim via brain pump showed similar effects, significant reduction of CSGP expression and in non-lesion, but no change in GFAP and MBP positive tissue area.</td>
</tr>
<tr>
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<tr>
<td>Pannu J. Neurochem, 2007.</td>
<td>Model: Female SD rats (225-250g), Weight drop contusion at T12 (40g cm force)</td>
<td>Atorvastain (AT) 5mg/kg/day gavage. Started 2h, 4h, or 6h PI, continued for 42d.</td>
<td>Behavioral Analysis 1. Saline (n=9/group) 2. AT (n=9/group) Histology/biochemistry 1. Saline (n=3 /group) 2. AT (n=3 /group)</td>
<td><strong>Behavioral:</strong> AT promotes significant locomotor recovery 6 weeks post-SCI. BBB scores were ~19 for each of the three AT treated groups and ~8 for controls; animals treated after 2h had the fastest recovery but all groups peaked around 19. <strong>Histologic/Biochemical/Physiological:</strong> 4h post injury, AT reduces the expression (mRNA) of iNOS, TNFα, and IL-1β. 6h post injury, AT reduces Rho activity 24h post injury, AT treatment promotes tissue sparing (H&amp;E stain), reduces neutrophil and macrophage infiltration, MMP 9 activity and expression, and Evans Blue extravasation. 5days post injury, AT reduced GFAP expression (mRNA).</td>
</tr>
<tr>
<td>Pannu J. Neurosci Res, 2005.</td>
<td>Model: Female SD rats (225-250g), Weight drop contusion at T12 (30g cm force)</td>
<td>Atorvastatin (AT) 5mg/kg/day gavage. Pretreatment 7d before SCI, given for 15d.</td>
<td>Behavioral Analysis 1. Saline (n=9/group) 2. AT (n=9/group) Histology/biochemistry 1. Saline (n=3 /group) 2. AT (n=3 /group)</td>
<td><strong>Behavioral:</strong> AT promotes significant locomotor recovery. At 15 days post-SCI, BBB scores were ~19 in AT treated and ~9 in controls. <strong>Histologic/Biochemical/Physiological:</strong> AT decreases secondary tissue damage. AT reduced iNOS, TNFα, and IL-1β mRNA expression (acute). AT reduced macrophage invasion, GFAP reactive astrocytes, TUNEL positive apoptotic cells (1 week) – Note: there was no quantification in these outcomes, only representative images were shown.</td>
</tr>
</tbody>
</table>

SCI: spinal cord injury; d: day; h: hour; w: week; PI: post-injury; q24h: interval 24 hours; T: thoracic vertebra; C: cervical vertebra; IP: intraperitoneal; IV: intravenous; SC: subcutaneous BBB: Basso, Beattie and Bresnahan locomotor test; MP: Methylprednisolone; SD rats: Sprague-Dawley rats; TNF: tumor necrosis factor
Figure 1.1 The Mevalonate Pathway
1.6 References


CHAPTER 2

A Novel Contusive Model

of

Unilateral Cervical Spinal Cord Injury

Using

The Infinite Horizon Impactor

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1 A version of this chapter has been submitted for publication. Jae H. T. Lee, Seth Tigchelaar, Elena B. Okon, Michael Maloon, Jie Liu, Anthea M. T. Stammers, Femke Streijger, Wolfram Tetzlaff, and Brian K. Kwon (2010) A novel contusive model of unilateral cervical spinal cord injury using the infinite horizon impactor.
2.1 Introduction

The paralysis caused by spinal cord injury (SCI) is a devastating condition for which there are currently no effective treatments. It is estimated that over a million individuals in the United States alone suffer spinal cord paralysis, with billions of dollars spent each year in medical costs alone (Christopher and Dana Reeve Foundation, 2009). SCI is typically caused when the spinal column fractures and/or dislocates, imparting a combination of contusive, compressive, and distractive forces on the cord. This initial mechanical insult causes the “primary injury” whereby axons, cell membranes, and blood vessels are disrupted. This is followed by a complex series of secondary pathophysiologic processes, such as excitotoxicity, ischemia, apoptosis, inflammation and oxidative stress, which exacerbate the injury (Beattie et al., 2002; Guth et al., 1999; Hulsebosch, 2002; Jones et al., 2005; Kwon et al., 2002; Kwon et al., 2004; Tator, 1995; Tator and Fehlings, 1991). In order to develop therapies in the laboratory for SCI, it is necessary to have a precise and reproducible preclinical injury model that reflects the pathology and pathophysiology of the clinical condition (Anderson and Stokes, 1992).

More than half of human SCIs are incomplete and the majority also occur in the cervical spinal cord (NSCISC, 2010). Despite the higher occurrence of SCI in the cervical level, most SCI research is currently performed in rodent models of thoracic SCI. While thoracic injury models have been extensively developed and generate reproducible injuries, the central pattern generator that mediates lower limb movement makes interpretation of locomotor recovery in rodent thoracic SCI studies difficult. In contrast, cervical injury models allow for the assessment of “local” or “segmental” motor recovery in the forelimbs. Given that regaining upper extremity function is a top priority for individuals living with cervical spinal cord injuries (Anderson, 2004), there is a compelling rationale for evaluating therapies in cervical injury models, where recovery of forelimb function can be used as an outcome measure.

The Infinite Horizon Spinal Cord (IH) impactor (Precisions Systems, Lexington, KY) is a commercially available spinal cord injury contusion device that is “force-controlled” (Figure 2.1). The researcher sets the intended force to be imparted and as the impactor tip plunges into the cord, a signal to stop and withdraw is triggered when it reaches that force. An immediate display of force and displacement over time are provided, allowing one to determine the biomechanical veracity of the injury. Originally, the IH Impactor was developed for use in a thoracic SCI model,
and it has been shown to produce consistent and reproducible contusion injuries in this paradigm (Scheff et al., 2003). It has rapidly become widely utilized in the SCI research community.

In this study, we describe the development of a novel, clinically relevant cervical unilateral contusion injury using the IH impactor. We describe the refinement of the biomechanical, functional and histological parameters of the injury model. The injury parameters established in these experiments generate a unilateral injury model with sustained functional deficits over 6 weeks and characteristic parenchymal damage to the unilateral spinal cord. This injury model will provide an additional preclinical tool that can be used to evaluate promising therapeutic agents before moving to clinical trials.
2.2 Material and Methods

All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. All surgeries and behavioral and histological evaluations were performed by a person blinded to the treatment.

2.2.1 Surgical Procedure

Sprague Dawley rats (Charles River Breeding Laboratories, n=207) weighing between 300 and 350 grams were anesthetized with 2% isoflurane in oxygen (1 L/min). Animals were placed in a stereotaxic frame (Kopf®, Tujunga, CA) and a 4 to 5 cm dorsal midline incision was made to expose the posterior vertebral elements from C3-C8. A laminectomy was performed on the left side at C5 to expose the left half of the spinal cord. A custom-built clamp which firmly grasps the vertebrae just ventral to the transverse processes was applied, stabilizing the spinal column from C4-C6 (Figure 2.1) (Choo et al., 2009). This clamping system allows one to position the spinal cord precisely under the impactor tip of the Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation, Lexington, KY), and also allows one to rotate the animal so that the trajectory of the impactor tip is at an angle to the spinal cord (Figure 2.2). With the animal positioned in the clamping system, a flat impactor tip (diameter: 1.5 mm, with rounded edges) was lowered until it was 4 mm above the dura. The impactor was then triggered to deliver a pre-determined force of either 75, 100 or 150 kdyne at 100 mm/s. The device immediately provides a graph of the actual reaction force and displacement experienced by the impactor tip during the impact (Figure 2.3).

Temperature was monitored with a rectal thermometer (TCAT-2LV Temperature Controller, Kopf, Tujunga, CA) and an incubator was used to maintain a body temperature of 37°C during and after the surgery until the animals were fully awake and mobilizing. A subcutaneous injection of buprenorphine (0.02 mg/kg, Temgesic®, Reckitt Benkiser Healthcare Ltd., UK) and 10 mL saline were administered just prior and for 2 days after the surgery.
2.2.2 Experimental Groups

Three experiments were carried out to assess the effect of impact force, impact angulations, and impact distance from midline. The first experiment evaluated contusion injuries induced by three different injury forces (75, 100, or 150 kdyne). The animals were in a neutral, un-rotated position, and the impactor tip was aimed 1.2 mm to the left of midline. The second experiment evaluated a single injury force (150 kdyne) aimed 1.2 mm to the left of midline, but with the animals rotated either 17.5°, 20.0°, 22.5°, or 25.0°, so that the impactor tip was striking the cord at different angles (Figure 2.2). In the third experiment, the animals were rotated 22.5° and a single injury force (150 kdyne) was utilized, but aimed either 1.0, 1.2, or 1.4 mm to the left of midline. Table 2.1 lists the experiments and numbers of animals per group.

2.2.3 Behavioral Outcome Measures

Baseline testing for all behavioral outcome measures was performed 1 week prior to the spinal cord injury. Postoperative behavioral assessments were performed 1, 2 and 4 weeks post-injury for experiment #1, and 2, 4, and 6 weeks post-injury for experiment #2 and #3.

*Horizontal Ladder Test.* As described previously (Soblosky et al., 2001), the animals began at the right end of the horizontal ladder apparatus and were videotaped moving from right to left so that the left forelimb was clearly visible to the high definition video camera (Sony HDR-SR12). A total of five runs across the ladder were recorded and the footage of each rat was later analyzed in slow motion to count the number steps and numbers of slips for both forelimbs. The number of slips for the left forelimb (ipsilateral to the SCI) was represented as the “percentage of ipsilateral forelimb errors”, calculated by dividing the number of forelimb errors by the total number of forelimb steps. [% ipsilateral forelimb errors = (ipsilateral forelimb errors / total number of ipsilateral forelimb steps) x 100].

*Cylinder Rearing Test.* Animals were placed into a clear topless cylinder measuring 30 cm in height and 30 cm in diameter (Schallert et al., 2000). As they vertically reached up on the cylinder wall, 20 exploratory performances with their forelimbs were recorded using a high definition video camera (Sony HDR-SR12). The footage was later analyzed frame by frame and the number of times the animal reached up and touched the wall with its left forelimb, right forelimb, or both
forelimbs at the same time was counted. To evaluate the forelimb use, we added the number of
times the animals reached up and touched the cylinder wall with left paw alone, or when it did so
with both left and right paws simultaneously. The frequency of injured forelimb usage was
represented as the “percentage of ipsilateral forelimb usage”, calculated by adding the number of
times the animal reached up and explored with either its left paw alone or with both paws
simultaneously, and dividing this by the total number of paw placements [% ipsilateral forelimb
usage = (left forelimb only + subsequent left and right forelimb simultaneously) / total forelimb
usage x 100].

*Modified Montoya Staircase Pellet Reaching Test.* Fine forelimb digit control was assessed using
the modified Montoya staircase in which animals crawl into a small space to reach and grasp food
pellets contained in wells at 7 differing depths (Montoya et al., 1991; Nikkhah et al., 1998). Before
the injury, animals were first acclimatized to the apparatus and food pellets (45 mg, Bio-Serv,
Frenchtown, NJ) by placing the apparatus in their home cage 2 weeks prior to injury. There were a
total of 14 pellets on each side (2 pellets in each well). For each time point that was assessed, the
animals were fasted for 20 hours before being placed in the staircase for 15 minutes. The number
of pellets retrieved from each well was counted afterwards. The “Ipsilateral percent retrieval” of
ipsilateral forelimbs was calculated by dividing the number of pellets retrieved by 14. [Ipsilateral
percent retrieval (%) = (number of pellets retrieved / 14) x 100].

*Grooming Test.* Animals were placed into a clear topless cylinder measuring 30 cm in height and
30 cm in diameter. A few drops of sterile saline were applied between the ears, and their grooming
response was recorded for 15 minutes using a high definition video camera (Sony HDR-SR12).
The footage was later analyzed to assign a grooming score developed by Gensel et al. (2006)
(Table 2.2). In brief, the score describes the forelimb range of motion while the rat is grooming.

**2.2.4 Histological Outcome Measures**

*White and Grey Matter Sparing.* The sections were stained with Eriochrome Cyanine (EC) as
described by Rabchevsky et al. (2001) counter-stained with Neutral Red, and then photographed on
a Leica DM5000B microscope with an 2.5x objective. Regions of intact white and grey matter on
both the ipsilateral and contralateral side of the spinal cord were manually traced and then
quantified using SigmaScan Pro version 5.0.0 (Systat Software Inc.). To take advantage of the
unilateral nature of the injury, the extent of white and grey matter sparing that was quantified on the injured side was expressed as a percentage of the uninjured side of the spinal cord on each section. The “epicenter” was defined as the cross-section with the least amount of white and grey matter sparing (i.e. the greatest extent of parenchymal damage). In cases where the parenchymal damage on the left side of the cord was extensive and crossed the midline over to the right side, we were still interested in the percentage of damage on the impacted (Left) side compared to the right side. Hence, for such cross sections we extrapolated what the “uninjured” white and grey matter sparing would be on the right side of the cord by tracing the white and grey matter on the right side as if there were no injury (Figure 2.4).

2.2.5 Statistics

All behavior and histological assessments were compared using the SPSS 13.0 t-test and One-way ANOVA test followed by Scheffe test, least significant difference (LSD) and Bonferroni for multiple comparisons. A p-value less than 0.05 was considered to be statistically different.
2.3 Results

2.3.1 Experiment 1: Effect of Different Impact Forces

In this experiment, the animals were in the prone, neutral position with no rotation (parallel to the horizontal plane) (Figure 2.2), and three different injury forces were tested: 75 kdyne (n=12), 100 kdyne (n=9), and 150 kdyne (n=2). Only two animals received 150 kdyne injuries, because the actual forces recorded in these two were 456 and 449 kdyne, with displacements of 1499 and 1569 µm respectively. In both animals, we observed a characteristic sharp “spike” on the force vs. time graphs, which indicated that the impactor was striking a stiff surface that caused a rapid increase in force (Figure 2.5). For the 9 animals that received 100 kdyne injuries, 5 animals revealed impact forces between 100 and 109 kdyne, and displacements between 1040 to 1428 µm, but the remaining 4 animals had impactor forces with the same sharp spike as seen with the 150 kdyne injuries. These 4 had impactor forces between 110 and 205 kdyne and displacements in the range of 1340 to 1499 µm. At 75 kdyne, 8 out of 12 animals had impact forces between 75 and 82 kdyne with displacements in the range of 846 to 1040 µm, while 4 out of 12 animals showed the same sharp spike as seen with the 150 kdyne injuries, with forces between 83 to 277 kdyne and displacements between 1393 to 1605 µm. We interpreted from the force vs. time graphs that the animals with a sharp spike had an injury in which the impactor tip had travelled through the spinal canal and struck the ventral bone, eliciting the rapid increase in force (Table 2.3).

Behavioral Outcome Measures

For the analysis of behavioral outcomes, we stratified the animals based on whether a spike had appeared on their force vs. time graphs, because we interpreted that the presence of a spike represented a substantial “deviation” from the expected biomechanics of the injury. The animals were therefore regrouped with the following injuries: 75, 75-Spike (S), 100, 100-S, and 150-S kdyne (all 150 kdyne injuries had this spike) to investigate the differences in functional recovery between the groups.

Horizontal Ladder Test. For this experiment, horizontal ladder testing was conducted at 1, 2, and 4 weeks post-injury. Compared to pre-injury baseline testing, all groups (regardless of the presence
or absence of a spike) demonstrated significant ipsilateral forelimb impairment at 1 week post-injury, as revealed by the substantial increase in forelimb placing errors (Figure 2.6). At this time-point, the 100 kdyne group demonstrated the greatest number of errors (23.82 ± 3.16), although all groups improved substantially at week 2. By 4 weeks, the 100 kdyne group made significantly more errors compared to all the animal groups with spikes on the force vs. time graph (100 kdyne: 8.28 ± 0.63, 75-S kdyne: 2.0 ± 0.4, 100-S kdyne: 1.7 ± 0.38 and 150-S kdyne: 3.1 ± 0.9 %, p<0.001). Interestingly, at this time point (4 weeks post-injury) the percentage of forelimb errors for the animals in the 75 kdyne group and those in the groups with spikes (100-S, 75-S and 150-S) had all returned back to baseline pre-injury levels (Figure 2.6).

**Grooming Test.** In all groups, the grooming score decreased significantly after the injury. Four weeks after injury, animals in the 75-S, 100-S and 150-S kdyne groups had average scores of 3 out of 5 (grooming above to eye all the way up to the front of ears), while the animals in the 75 and 100 kdyne groups (without spikes) had average scores of 2 out of 5 (grooming below the level of the eyes) (Figure 2.7).

**Histological Outcomes**

Histologically, the corticospinal tract in the dorsal column appeared to be unaffected, while the rubrospinal tract in the dorsolateral funiculus appeared to be severely damaged in all groups. However, the groups with a sharp spike displayed a very different pattern of injury. Generally, the 75-S, 100-S and 150-S kdyne groups had smaller lesion area, and the lesion was located more laterally compared to 75 and 100 kdyne groups (without spikes) (Figure 2.8).

Based on the behavioral results of Experiment 1, we concluded that the presence of spikes on the force vs. time graphs was a clear deviation from the expected biomechanical parameters of the injury. We postulated that the spike was related to the impactor tip suddenly striking the bone on the ventral aspect of the spinal canal, and that the force at that moment was not being imparted as intended to the left half of the cord. Based on the extensive histological damage to the lateral aspect of the cord in these animals, we hypothesized that the impactor tip is slipping off the lateral edge of the cord and “pinching” the lateral rim of the cord between it and the bone, which it strikes with significant force (>100 kdyne). Because the impactor tip has slid laterally off the cord in these injuries, the medial parenchyma is spared, which then may explain why these animal groups had
less forelimb impairment, as evidenced by the fewer errors on the horizontal ladder and better performance on the grooming test. We also concluded that the 75 kdyne injury was unlikely to be severe enough to cause a sustained forelimb impairment (given the fact that the performance of the animals at 4 weeks on the horizontal ladder had recovered back to pre-injury levels). Furthermore, with the animals in the 100 kdyne injury group recovering at 4 weeks post-injury to only making 8% forelimb errors, we felt that there would be virtually no room to display any improvement with a treatment (i.e. a ceiling effect). Hence, we concluded that in subsequent experiments, we should employ the 150 kdyne force (to ensure a severe enough injury) and then take steps to avoid the impactor from what appeared to be a “bottoming out” on the ventral bone of the spinal canal.

2.3.2 Experiment 2: Effect of Different Angles of Rotation for Impact Trajectory

To prevent the impactor tip from striking the ventral side of the spinal canal, the animals were rotated at four different angles: 17.5°, 20.0°, 22.5° and 25.0° so as to change the trajectory of the impactor tip from a vertical plane to a more ‘lateral-to-medial’ plane (Figure 2.2). One hundred and fifty-six animals were used for this experiment, with the force kept constant at 150 kdyne. We imposed a force threshold of 165 kdyne (i.e. 10% greater than the intended force of 150 kdyne), above which animals were excluded.

In keeping with our hypothesis about why the force-spikes were occurring, we noted that as the animal’s angle of rotation increased (and the trajectory of the impactor tip moved away from vertical), fewer force-spikes occurred. In the 22.5° group, forces were below 165 kdyne 78.8 % of the time and in the 25.0° group 85.7 % of the time. The forces observed when changing the trajectory of the impact to 17.5°, 20.0°, 22.5° and 25.0° off of vertical are presented in Table 2.4.

Behavioral Outcome Measures

As a high proportion of the forces observed in the 17.5° and 20.0° groups exceeded 165 kdyne (Table 2.4), we felt that these injury parameters were not suitable for future studies. Hence, we restricted our assessment of behavioral outcomes to the 22.5° and 25.0° groups at 2, 4 and 6 weeks post-injury.
Horizontal Ladder Test. The injury caused significant impairment on forelimb placement compared to baseline pre-injury placement in the 22.5° and 25.0° groups. There were substantial increases in percentage ipsilateral forelimb errors at 2 weeks. At this time point, the percent errors were 25.53 ± 3.90% and 28.27 ± 3.79% for the 22.5° and 25.0° groups, respectively. These deficits were maintained at 4 weeks (22.5°: 22.51 ± 3.83% and 25.0°: 21.30 ± 2.98%). At 6 weeks, the percent error decreased slightly to 18.01 ± 2.72 and 15.29 ± 2.28 for the 22.5° and 25.0° group, respectively. There was no significant difference between the 22.5° and 25.0° groups at any time point (Figure 2.9A).

Cylinder Rearing Test. The percentage of ipsilateral forelimb usage reduced dramatically after the injury. Generally, the animals in the 25.0° group showed slightly less ipsilateral forelimb usage compared to 22.5° group, but there were no statistically significant differences between the two groups at any time point. The percentage of ipsilateral forelimb usage of the 22.5° group was reduced to 20.04 ± 9.70% at 2 weeks post-injury, and the deficit was maintained at 19.39 ± 5.74% and 14.92 ± 6.80% for 4 and 6 weeks, respectively. For the 25.0° group, the percentage forelimb usage decreased dramatically to 3.17 ± 3.17% at 2 weeks, recovered slightly to 8.03 ± 5.69% at 4 weeks, and maintained usage at 7.69 ± 5.71% by 6 weeks (Figure 2.9B).

Modified Montoya Staircase Test. Prior to injury, animals retrieved all 14 pellets on each side of the staircase. The percentage of pellet retrieval decreased significantly after the injury. At 2 weeks post-injury, animals in the 22.5° group retrieved 35.71 ± 6.39 %, whereas the 25.0° group retrieved 25.39 ± 2.69 % of the total pellets. Four weeks after injury, both 22.5° and 25.0° groups maintained deficits at 32.65 ± 4.37 % and 25.39 ± 3.59 %. At 6 weeks post-injury, animals in the 22.5° group retrieved 28.57 ± 3.82 % of pellets, but those in the 25.0° group recovered only 16.43 ± 3.85 % of total pellets. The two groups were significantly different at 6 weeks (p=0.047, Figure 2.9C).

Histological Outcomes

In this experiment, both corticospinal and rubrospinal tracts were injured on the ipsilateral side, but the extent of injury frequently crossed over to the right side of the cord, damaging parts of the contralateral corticospinal tract in majority of animals in both groups (Figure 2.9D). In addition,
the extent of the damage at the lesion epicenter were enlarged more ventrally compared to Experiment #1, completely destroying grey and white matter area on the injured side.

In Experiment 2, we hypothesized that by rotating the angle of the animals and changing the trajectory of the impactor tip through the cord, we could avoid the impactor slipping off the lateral side of the cord and cause more consistently severe parenchymal damage (with concomitantly increased functional impairments). We definitely observed greater white and grey matter damage as compared to animals in Experiment #1 (with no rotation), and the functional assessments suggest that there are significant forelimb impairments sustained throughout the experimental period. We concluded that setting the force at 150 kdyne and angling the animal at 22.5° or 25.0° resulted in substantial damage to the grey and white matter on the injured side and produced sufficient and sustained functional deficits. However, we observed that the histologic damage was frequently not contained on the intended side of the spinal cord and extended to the contralateral side.

2.3.3 Experiment 3: Effect of Moving Impact Away From Midline

In this experiment, we sought to determine if moving the impact site away from midline would prevent the parenchymal damage from extending to the contralateral side of the spinal cord. Based on Experiments 1 and 2, we selected a force of 150 kdyne, and an angle of 22.5°. We then moved the impactor tip to three different lateral settings away from midline: 1.0, 1.2, or 1.4 mm (Figure 2.10). Twenty-four animals were used in this study (8 per group).

Behavior Outcome Measures

Functional recovery was assessed using the horizontal ladder test, cylinder rearing test, grooming test, and modified Montoya staircase test at 2, 4 and 6 weeks post-injury.

**Horizontal Ladder test.** Following the injury, animals in the 1.0, 1.2, and 1.4 mm groups all demonstrated marked increases in the percentage of forelimb errors while traversing the horizontal ladder; however, no significant differences were observed between the groups at 2, 4 and 6 weeks post-injury. At 6 weeks post-injury, the percentages of ipsilateral forelimb errors were 24.38 ± 4.42%, 25.36 ± 6.43% and 23.44 ± 2.73% for the 1.0, 1.2, and 1.4 mm groups, respectively (Figure 2.11A).
Cylinder Rearing test. The percentage of ipsilateral forelimb usage during exploration decreased significantly after SCI, but there were no statistically significant differences between the 1.0, 1.2 and 1.4 mm groups. At 6 weeks, the percentages of ipsilateral forelimb usage were 16.58 ± 5.89%, 15.01 ± 4.23% and 24.90 ± 6.13 % for the 1.0, 1.2, and 1.4 mm groups, respectively (Figure 2.11B).

Grooming test. There were dramatic decreases in the grooming scores after injury, but again, there were no statistically significant differences between the 1.0, 1.2 and 1.4 mm groups. At 6 weeks, the grooming scores were 2.13 ± 0.22, 1.75 ± 0.14 and 2.00 ± 0.21 for the 1.0, 1.2, and 1.4 mm groups, respectively (Figure 2.11C).

Modified Montoya Staircase test. For all groups, the number of pellets retrieved decreased dramatically after the injury. At 2 weeks post-injury, animals in the 1.4 mm group collected significantly more pellets compared to those in the 1.0 and 1.2 mm groups (p=0.002), but at 6 weeks post-injury all three groups were comparable in their retrieval performance (Figure 2.11D).

Histological Outcome

In the 1.0 mm group, 7 out of 8 animals had obvious parenchymal damage expanding to the contralateral side (87%). In the 1.2 mm group, 3 out of 8 animals had damage extending to the contralateral side (38%). In the 1.4 mm group, 2 out of 8 animals had damage spreading to the contralateral side (25%). As the aim moved away from the midline, the damaged appeared to move laterally as well (Figure 2.12).

White matter and Grey matter sparing. There were no statistical differences between the 1.2 and 1.4 mm groups in white matter and grey matter sparing; however, the 1.0 mm group had significantly less white matter sparing compared to the 1.2 and 1.4 mm groups at the epicenter, and at the sections 400 µm and 800 µm caudal to the epicenter (p=0.004, p=0.005 and p=0.002, respectively, Figure 2.13A). In addition, the 1.0 mm group had statistically less white matter sparing compared to 1.2 mm group 1200 µm caudal to the epicenter (p=0.026, Figure 2.13A). For grey matter sparing, the 1.0 mm group had statistically less tissue sparing at the epicenter and caudal 400 µm (p=0.015 and p=0.009, Figure 2.13B). When adding the sections up to provide a gross estimation of the “cumulative spread” of white and grey matter sparing 2000 µm rostral and caudal to the epicenter, the 1.0 mm group had significantly less white and grey matter compared to 1.2 and 1.4 mm groups (p=0.045 and p=0.021, Figure 2.13C, D).
In this experiment, we show that functionally there were no differences between 1.0, 1.2 and 1.4 mm setting. Histologically, as we moved the aim laterally, the injury was more likely to be contained on the injured side. Generally, 1.0 mm group had less white and grey matter sparing compared to 1.2 and 1.4 mm groups. In order to compare the spared white and grey matter of the injured side between groups, we extrapolated the uninjured white and grey matter on the contralateral side and used this area to normalize the injured side to express them as a percent spared matter (Figure 2.4). In addition, we compared these normalized values to actual spared white and grey matter area of the injured side only (without incorporating the contralateral side) and the extent of white and grey matter sparing at each level and the amount of cumulative white and grey matter sparing were almost identical.
2.4 Discussion

In this paper, we describe the systematic development of a cervical unilateral contusion model using the Infinite Horizon Spinal Cord impactor at a force of 150 kdyne, an angle of 22.5° off of vertical, and a lateral aim of 1.4 mm from the midline. With these settings, we were able to produce sustained behavioral deficits in the ipsilateral forelimb with parenchymal damage largely contained to the ipsilateral side, where it appeared that both the rubrospinal and corticospinal tracts incurred significant damage. The development of the model occurred in a series of three experiments. We first observed that impactor forces below 150 kdyne did not produce sufficient and sustained functional deficits, and that a vertical impactor trajectory frequently resulted in spikes in the force vs. time curves, indicating that the impactor tip was hitting bone on the ventral side of the spinal canal. We therefore rotated the animals to change the trajectory of the impactor to 17.5°, 20.0°, 22.5° or 25.0° off of the vertical midline. Using a 150 kdyne force, the increased angulation tended to resolve the problem of force spikes, but extension of the parenchymal damage to the contralateral side of the cord was observed. Finally, we aimed the impactor at three different distances off of midline, 1.0, 1.2 and 1.4 mm, using a 150 kdyne force and an angle of 22.5°. We observed that there were no behavioral differences between the 1.0, 1.2 and 1.4 mm settings, but as the aim moved laterally, it was more likely that the gross parenchymal damage could be contained on the ipsilateral side. We therefore arrived at our current unilateral contusion settings of a 150 kdyne injury delivered at 22.5° off of vertical, with the impactor tip aimed 1.4 mm to the left of midline.

The rationale for making cervical injury models available for the testing of SCI therapies is clear: the majority of individuals suffer cord injuries in the cervical spine, upper extremity function is paramount to these individuals, and clinical trials of novel neuroprotective or neuroregenerative interventions are increasingly focusing on cervical SCI patients in order to use segmental motor recovery as an outcome measure. Injury to the cervical spinal cord can occur via laceration, compression, or contusion. Amongst these injury models, contusion and compression injuries best represent the pathophysiological process observed in human SCI (Gensel et al., 2006; Kwon et al., 2002; Kwon et al., 2010). According to a recent survey of the SCI research community, 72% of the 324 respondents agree that contusion injury is the most clinically relevant injury model of SCI (Kwon et al., 2010).
Since Reginald Allen’s description of the first experimental weight-drop device for generating spinal cord injuries in the laboratory setting (Allen, 1911), a number of contusion devices have been developed in an effort to optimize reproducibility and to generally simulate the pathology of human injury (Kwon et al., 2002). The New York University impactor uses electromechanical components to measure injury displacement and velocity during weight drop (Basso et al., 1996; Gruner, 1992). Here, injury severity is dictated by the height from which the weight is dropped. In contrast, in the Ohio State University (OSU) impactor and the multimechanism injury system designed by Choo et al. (2009), the maximum displacement of the spinal cord is determined, and the force imparted to the cord is then measured. The IH impactor is distinct in that the user dictates the force applied, and then the displacement is measured. While each of these systems (weight-drop versus displacement-control versus force-control) has its theoretical advantages, the relative ease of use and commercial availability of the IH impactor has made it increasingly popular in recent years. In fact, we had previously established a unilateral cervical contusion SCI model with the OSU impactor, and have utilized it to evaluate an acute neuroprotective intervention (Lee et al., 2010, in Press). However, we made the decision to switch to the IH impactor because of its ease of use, and – from a practical perspective - the availability of technical support from the manufacturer. This manuscript reveals that optimizing and refining the injury technique with this impactor was not such a straightforward endeavor.

Experiment 1 was conducted in a similar manner to our previous work, using the OSU impactor for unilateral cervical SCI: with the tip vertical to the spinal cord, approximately 1.2 mm to the left of midline, and with various injury severities – in this case, 75, 100, and 150 kdyne. We immediately observed very distinct and sharp “spikes” in the force vs. time curves with all injury severities, and it was obvious that we were not achieving injuries with precise force control. While it would be difficult to prove conclusively, we postulated that this “spike” was the result of the impactor slipping off the left lateral edge of the spinal cord, or the spinal cord “squirting off” to the right. In either scenario, the descending impactor tip would not be subjected to increasing reaction forces and would not reach its target force until suddenly “running into” the ventral, bony surface of the spinal canal – resulting in a sudden “over-shooting” of the target force. The maximal excursion of the tip is 15 mm, and so without it experiencing the target force, it will most certainly keep descending until it reaches something hard (in this case, most likely the bone on the ventral spinal canal). With this, either the impactor tip would strike the bony spinal canal unimpeded, or a
variable amount of the very lateral border of the spinal cord might be caught and crushed between the impactor tip and bony spinal canal. In either scenario, the impactor tip “glances off” the spinal cord, misses the intended target, and the overall force applied to the actual spinal cord would be low. Consistent with this, the histological analysis revealed reduced parenchymal damage in these animals with the sudden force spikes, and the damage also appeared to be very laterally placed (Figure 2.8). Additionally, after regrouping the animals according to the occurrence of the abnormal contusion, we found that all animals with this sudden force spike had negligible functional deficits at 4 weeks.

Further support for our proposed mechanism for the force spikes comes from Experiment 2, where we angled the animals to change the trajectory of the impactor tip. By having the impactor tip come in more perpendicular to the spinal cord, we felt that the impactor tip was less apt to “slip off” to the left (or conversely, for the cord to get “squirted off” to the right, or a combination of both). The fact that we observed fewer “spikes” as the angle was increased to 25.0° is consistent with this proposed mechanism. With this angulation, the impactor tip is actually more perpendicular to the curved surface of the spinal cord, and is therefore plunging more directly into the parenchyma. In doing so, the tip more consistently reaches its threshold force of 150 kdyne, then reverses its course, producing the more typical force vs. time curve without a sudden spike.

From a technical perspective, we should also note that significant modifications were made in the method for clamping the animals and securing them prior to impact (Figure 2.1). Whether these modifications contributed to the “spikes” is difficult to say, but the clamping system developed certainly holds the animals very rigidly during the impact. As the IH impactor was originally developed for thoracic injuries, the clamps provided with the device are designed to hold the spinous processes of the thoracic spine, which are substantially larger than in the cervical spine. The inability to hold the animals firmly with these clamps and prevent subtle movement at the time of injury should theoretically not influence the injury mechanics significantly, as the impactor is designed to impart the same force each time. A previous pilot study from our laboratory indicated that in fact, even with a force controlled impactor, subtle movements during the injury due to breathing or clamp failure resulted in aberrant degrees of parenchymal damage and unpredictable functional deficits (unpublished data). To improve upon the consistency of our injuries, we stabilized the animals with a custom-built clamping system that firmly grasps the transverse
processes of the cervical spine (Choo et al., 2009). The improvement in consistency of injury with such a clamping system is reported in Choo et al. (2009).

Finally, in Experiment 3, we moved the impact site either 1.0, 1.2, or 1.4 mm to the left of midline in an effort to contain the degree of obvious parenchymal damage to the left side of the cord. Expectedly, a more medial injury (1.0 mm) resulted in damage extending to the right side of the cord, and a more lateral injury (1.4 mm) resulted in better containment on the left side. Whether containment on the one side of the cord is absolutely essential could be debated, particularly given that the behavioral outcomes were not statistically different between the 1.0, 1.2, or 1.4 mm groups in any of the functional tests performed. In our previous work with the OSU impactor, the impactor tip was aimed vertically with the animal in neutral rotation, and we did not observe extension of the injury to the right side. This enabled us to express the extent of sparing on the left side as a percentage of that on the right. When it occurred, extension of the damage to the right side of the cord was minimal, and we were thus able to fairly easily extrapolate what the contralateral extent of white matter and grey matter would be (Figure 2.4).

With respect to the intended target of the injury, we hoped to injure both the corticospinal (CST) and rubrospinal (RST) tracts, as these both play a role in forelimb function in rodents (Whishaw et al., 2003). In Experiment 1 with the animal in neutral rotation, the lateral nature of the injury appeared to have spared the dorsal CST, and this may have contributed to the negligible functional impairments in these animals (Figure 2.6, 2.7 and 2.8). We hypothesized by angling the trajectory of the impactor, we would be more likely to damage both the CST and RST, and therefore induce a more substantial and sustained functional impairment. Of course, this angulation also helped us resolve the issue of the force spikes. The relationship between CST and RST damage in our model and the functional impairments is only postulated – retrograde and anterograde tracing studies would be needed to quantify RST and CST sparing (if any) and correlate these with functional recovery to strengthen this association.

In our study, functional deficits were assessed with the horizontal ladder test, cylinder rearing test, grooming test and Montoya staircase test. Both the horizontal ladder test and cylinder rearing are valuable assessments after cervical injury models (Jones and Schallert, 1992; Liu et al., 1999; Schallert et al., 2000; Whishaw et al., 2003). The horizontal ladder test forces the animals to use both their injured and uninjured forelimbs to get across the ladder, and hence, the test measures the compensatory and adaptive function of the forelimb. During the pre-injury training, animals
typically will “grab” or place their forepaw on the bars with their digits while crossing the ladder. After severe or moderate cervical unilateral contusions, most of this motor function is abolished, and animals are no longer able to consistently place or grasp the rungs (Gensel et al., 2006; Soblosky et al., 2001). The cylinder rearing test examines recovery naturally by analyzing voluntary forelimb usage. Typically, the use of the injured forelimb while exploring is dramatically reduced after injury. The loss of these functions are likely related to a combination of both axonal disruption and to the eradication of motor neurons at the lesion epicentre, which innervate such muscles as the deltoid, biceps, extensor carpi radialis longus and the extensor carpi radialis brevis muscles (McKenna et al., 2000). The grooming test, like the cylinder rearing test, examines the gross natural behavior of the animals. The modified Montoya staircase evaluates the digit functions, or fine control, of the forelimbs. Surprisingly to date, there is only one study that has utilized the modified Montoya’s staircase test in cervical SCI (Lee et al., 2010). Together, these tests evaluate both the fine and gross components of the overall forelimb functions.

Other studies have also described cervical contusion models, which have typically been devised with some modification to a pre-existing thoracic contusion device (Table 1.1, 1.2) (Gensel et al., 2006; Sandrow et al., 2008; Soblosky et al., 2001). Previous work from our laboratory used the Ohio State University impactor at 1.5mm displacement to test unilateral cervical contusions (with a maximum peak force of 200 kdyne) (Lee et al., 2010). Sandrow et al. utilized the IH impactor at force of 200 kdyne and resultant displacements of 1.6 to 1.8 mm and then assessed behavioral outcomes with the forced locomotion test, forelimb open field locomotion, grip strength test and grid walk test (2008). Gensel et al. (2006) used a MASCIS/New York University impactor using 10 g at 6.5 mm and 12.5 mm height and assessed behavioral outcome with the grooming test, horizontal ladder test, cylinder rearing test and Semi-Automated Walkway test (Catwalk test). Soblosky et al. (2001) used a modified Allen’s weight drop device (10.5 g) to injury animals at 5.00, 2.50 or 1.25 mm heights on a 20.0° angle, and evaluated the horizontal ladder test and cylinder rearing test to assess behavioral recovery. It is difficult to compare our injury model to the study by Sandrow et al. (2008), since none of functional tests overlap with our current study. When we compare the functional outcomes to other studies, our current IH injury model is generally more severe compared to other cervical unilateral contusion reports. For the horizontal ladder test, Soblosky et al. (2001) reported the total number of slips without the total number of steps. The ipsilateral percent error at 6 weeks
post-injury for the our current model was about 25% for both 22.5° and 25.0° angles, compared to study by Lee et al. (2010) and Gensel et al. (2006), which reported errors in the range of 10 – 15%. For the cylinder rearing test, our 15-20% of ipsilateral forelimb usage for the 22.5° angle was comparable to that reported with a 5.0 mm weight drop (Soblosky et al., 2001). Comparing the behavioral deficits that resulted from the NYU impactor, Gensel et al. (2006) reports a complete abolishment of ipsilateral forepaw use for the 12.5 mm height. We also report no ipsilateral forepaw use for 25.0°, suggesting that the two injury models are comparable in this regard. For the grooming test, our animals scored less than the injury groups reported by Gensel et al. (2006).

Histologically, the injury model presented here generally induces greater parenchymal damage as compared to other cervical hemicontusion injury models. The rostral and caudal extension of our injury was 56 mm, as compared to 40 mm in Lee et al. and 36 mm in Gensel et al. studies using the OSU impactor and NYU impactor, respectively (Gensel et al., 2006); Lee et al., 2010). At the lesion epicenter, we found about 20% grey matter spared in our injury model, as compared to Lee et al. (2010) at 10%, Gensel et al. (2006) at 20 – 50% and Soblosky et al. (2001) at 31 – 99%. For white matter sparing, the injury model presented here left about 20% tissue remaining at the epicenter compared to 30% in Lee et al., (2010), 5 – 10% in Gensel et al., (2006) and 18 – 62% in Soblosky et al., (2001) at the lesion epicenter. In the study, by Lee et al., (2010) the rubrospinal tract suffered significant injury, but the corticospinal tract often appeared intact. Gensel et al. (2006) reported partial damage to the corticospinal tract and complete destruction of the rubrospinal tract for both height settings. Soblosky et al. (2001) report partial damage to the rubrospinal tract, but no injury to the corticospinal tract. These reports further enforce the importance of injuring both descending tracts in order to produce sufficient functional deficits (Alstermark et al., 1989). It is also worth to note that in Gensel et al. (2006) and Soblosky et al. (2001), injuries also extended to the contralateral side. Again, the importance of this issue of extension to the opposite side in our model is debatable, given that there were no behavioral differences between the injuries aimed 1.0, 1.2, and 1.4 mm off of midline, but it would be desirable contain the injury to ipsilateral side, since we use the contralateral side as control.

In conclusion, we report the development of a unilateral contusion injury, and hope to provide sufficient detail about its development and technique. Others who wish to study preclinical SCI therapies may employ such a model, using an impactor device that is widely available (the
Infinite Horizon impactor). We are currently utilizing the model to evaluate neuroprotective interventions, with the hope of providing important preclinical evidentiary support for specific treatments prior to human translation.
Table 2.1 Experimental Groups.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Force</th>
<th>Rotation</th>
<th>Distance from Midline</th>
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<tbody>
<tr>
<td>- Effect of different impact forces</td>
<td>75 kdyne (n=12) 100 kdyne (n=9) 150 kdyne (n=2)</td>
<td>0°</td>
<td>1.2 mm</td>
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<tr>
<td>Experiment 2</td>
<td>150 kdyne</td>
<td>17.5° (n=6) 20.0° (n=35) 22.5° (n=80) 25.0° (n=35)</td>
<td>1.2 mm</td>
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<td>- Effect of different angles of rotation for impact trajectory</td>
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</tr>
<tr>
<td>Experiment 3</td>
<td>150 kdyne</td>
<td>22.5°</td>
<td>1.0 mm (n=8) 1.2 mm (n=8) 1.4 mm (n=8)</td>
</tr>
<tr>
<td>- Effect of changing impact distance away from midline</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Grooming Score Description.

<table>
<thead>
<tr>
<th>Groom Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Animal is unable to contact any part of the face or head</td>
</tr>
<tr>
<td>1</td>
<td>Forepaw touches the underside of the chin and/or the mouth area</td>
</tr>
<tr>
<td>2</td>
<td>Forepaw contacts the area between the nose and the eyes</td>
</tr>
<tr>
<td>3</td>
<td>Forepaw contacts the eyes and the area up to the front of the ears</td>
</tr>
<tr>
<td>4</td>
<td>Forepaw contacts the front but not the back of the ears</td>
</tr>
<tr>
<td>5</td>
<td>Forepaw contacts the area of the head behind the ears</td>
</tr>
</tbody>
</table>
Table 2.3 Results of Different Impact Forces for Experiment 1.

<table>
<thead>
<tr>
<th>Force Level (kdyne)</th>
<th>Spike vs. No Spike on Force vs. time graph</th>
<th>Resultant Force</th>
<th>Displacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 (n=12)</td>
<td>n=8 No Spike 75 - 82 kdyne</td>
<td>846 - 1040 µm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=4 Spike 83 - 277 kdyne</td>
<td>1393 - 1605 µm</td>
<td></td>
</tr>
<tr>
<td>100 (n=9)</td>
<td>n=5 No Spike 100 - 109 kdyne</td>
<td>1040 - 1428 µm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=4 Spike 110 - 205 kdyne</td>
<td>1340 - 1499 µm</td>
<td></td>
</tr>
<tr>
<td>150 (n=2)</td>
<td>n=2 Spike 449, 456 kdyne</td>
<td>1499, 1569 µm</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4 Results of Different Angles of Rotation

<table>
<thead>
<tr>
<th>Angles</th>
<th>n=156</th>
<th>≤165 kdyne</th>
<th>&gt;165 kdyne</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.5°</td>
<td>6</td>
<td>50.0%</td>
<td>50.0%</td>
</tr>
<tr>
<td>20.0°</td>
<td>35</td>
<td>62.9%</td>
<td>37.1%</td>
</tr>
<tr>
<td>22.5°</td>
<td>80</td>
<td>78.8%</td>
<td>21.2%</td>
</tr>
<tr>
<td>25.0°</td>
<td>35</td>
<td>85.7%</td>
<td>14.3%</td>
</tr>
</tbody>
</table>
Figure 2.1 Infinite Horizon Injury Device.  A. Infinite Horizon impactor.  B. Front view and height of frame (box from A).  C. Close-up image of the clamp for holding cervical spine (from white box in panel B).
Figure 2.2 Illustration of Various Spinal Cord Rotation and Frame Set Up Top: Schematic and illustration of spinal cord rotated to 0° (neutral – as in Experiment 1), then 17.5°, 20.0°, 22.5° and 25.0° (as in Experiment 2). Bottom: Corresponding frame set up for the different angles.
Figure 2.3 Representative Force and Displacement Graphs for Infinite Horizon Impactor. The arrow indicates the time that the impactor tip has reached 20 kdyne and the point when the recording of displacement is initiated. The actual force is read from the peak of the force vs. time curve and the corresponding displacement is calculated. A typical 75 kdyne contusion, Left: displacement vs. time graph, Right: force vs. time graph. The actual displacement was 934 µm, and the peak force achieved 75 kdyne.
**Figure 2.4 Illustration of Spared White (W) and Grey (G) Matter Tracing on the Contralateral Side.** Left: Uninjured contralateral spinal cord. Right: When the damage extends to the right side, we have extrapolated the contralateral spared white and grey matter area from the midline.
Figure 2.5 Examples of Force Spikes. Abnormal force vs. time graph. A. Set force at 75 kdyne (Notice the 277 kdyne “spike” that occurs just before the force reaches 75 kdyne). B. Set force at 100 kdyne. The force diminishes from 80 to 30 kdyne and then a 205 kdyne spike occurs. The diminished force indicates that the impactor tip has slipped off the spinal cord. C. Set force at 150 kdyne. Notice the force rises steadily, and then at 140 kdyne, a dramatic “force spike” (449 kdyne) occurs. Our interpretation is that the impactor tip runs out of distance before reaching the desired force and hits the ventral surface of the bony spinal canal (boxes on graph).
Figure 2.6 Experiment 1: Horizontal Ladder Test. Animals in the 100 kdyne group made significantly more errors compared to the 150-Spike (S) kdyne group at 1 week (*). At 4 weeks, the animals in the 100 kdyne group made statistically more errors compared to those in the 75-S, 100-S and 150-S kdyne groups (^).
Figure 2.7 Experiment 1: Grooming Test. Animals in the 75 and 100 kdyne groups scored significantly lower (score: 2) compared to 75-S, 100-S and 150-S kdyne groups (score: 3).
Figure 2.8 Representative Images of Spinal Cord in Neutral Rotation (Experiment 1), with Impactor Set for 100 kdyne Injury. Left: contusion with force spike (actual force: 205 kdyne). When a force spike occurs on the force vs. time graph, there were less white and grey matter injury compared to normal injuries. Right: contusion with normal force (actual force: 100 kdyne).
Figure 2.9 Experiment 2: Behavioral Assessments for Impactor Angulation of 22.5° and 25.0° (150 kdyne, Aimed 1.2 mm Left of Midline): A. Horizontal ladder test. B. Cylinder rearing test. C. Modified Montoya staircase. Animals at 22.5° retrieved significantly more pellets compared to animals at 25.0° at 6 weeks post-injury (28.57 ± 3.82 % vs. 16.43 ± 3.85 %, p=0.047). D. Representative image of injury crossing over to the contralateral side at 22.5°.
Figure 2.10 Illustration of Aiming the Impactor Tip to the Left of Midline 1.0, 1.2 and 1.4 mm (Experiment 3). The lateral aim was set by taking advantage of the measurements on the horizontal adjustment knob that moves the stage medially and laterally relative to the animal.
Figure 2.11 Experiment 3: Behavioral Assessments for 1.0, 1.2 and 1.4 mm: A. Horizontal ladder test. B. Cylinder rearing test. C. Grooming test. D. Modified Montoya staircase test. There were no statistical differences in behavioral outcome at 6 weeks post-injury with the impactor tip striking the cord 1.0, 1.2 or 1.4 mm away from midline.
Figure 2.12 Representative Images of the Injury with Impactor Tip Striking the Cord 1.0, 1.2 or 1.4 mm off Midline. The tip trajectory is angled 22.5° off vertically, and the force is 150 kdyne.
Figure 2.13 Histological Analysis A. Percent spared white matter: There was a significant difference between 1.0 mm group compared to 1.2, 1.4 mm group at the epicenter, c400 µm and c800 µm and a difference between 1.0 mm and 1.2 mm group at c1200 µm. B. Percent spared grey matter: There was a significant difference between 1.0 mm group compared to 1.2 and 1.4 mm group at the epicenter and c400 µm. C. Cumulative white and grey matter (from r2000 µm to c2000 µm) Left: Cumulative white matter, Right: Cumulative grey matter. There was a significant difference between 1.0 mm group compared to 1.2, 1.4 mm groups for cumulative white and grey matter (p=0.045 and p=0.021).
2.5 References


CHAPTER 3

Lack of Neuroprotective Effects of Simvastatin and Minocycline in a Cervical Unilateral Contusion Model.

3.1 Introduction

Spinal cord injury resulting in sudden paralysis has not only a catastrophic impact on the individual patient, but also has dramatic societal and economical implications. Each year, there are over 10,000 North Americans that sustain traumatic spinal cord injuries, adding to over 1 million individuals estimated to already be living with spinal cord paralysis (Christopher and Dana Reeve Foundation, 2009). While this has traditionally been seen as an injury of youth, a bimodal age distribution has emerged in the past decade, with peaks in incidence in both young individuals and in those over the age of 65 (van den Berg et al., 2010). During the lifetime of an individual with SCI, financial requirements for care are estimated to be between $1 million and $3 million, depending on the severity of the injury and the annual cost to the health care system for SCI treatment is approximately 40 billion annually (Christopher and Dana Reeve Foundation, 2009).

Currently, there are no convincingly efficacious treatments to improve the neurologic outcome after acute human SCI. While high dose methylprednisolone remains a “treatment option” for acute SCI, rising skepticism around the drug’s neuroprotective efficacy and mounting concerns regarding its safety have prompted many physicians to abandon it (Hurlbert and Hamilton, 2008). Of course, this has stimulated intense scientific research efforts in preclinical animal models to develop new neuroprotective treatments that will hopefully minimize secondary damage and improve neurologic outcome. To expedite the process of bench-to-bedside translation, much attention has been paid recently towards the neuroprotective properties of pharmacological agents that are already in current clinical use (albeit typically for unrelated medical conditions) and thus have well-documented dosing and safety profiles in human patients. These include commonly used drugs such as minocycline, erythropoietin, riluzole, magnesium, estrogen, progesterone, atorvastatin, and ibuprofen (Kwon et al., 2010b).

Minocycline, a semi-synthetic tetracycline antibiotic commonly used to treat acne, has been studied previously by numerous labs as a potential neuroprotective agent after acute thoracic SCI. A number of investigators have reported on minocycline’s neuroprotective effects (Lee et al., 2003; Piao et al., 2003; Teng et al., 2004; Tikka and Koistinaho, 2001; Yrjanheikki et al., 1999; Yune et al., 2007; Zhu et al., 2002) and have found it to enhance behavioral recovery after models of thoracic SCI (Festoff et al., 2006; Ha et al., 2008; Lee et al., 2003; Marchand et al., 2009; Stirling et al., 2004; Wells et al., 2003; Yune et al., 2007). Promising results with minocycline in 2003
and 2004 stimulated the initiation of a prospective randomized pilot study for minocycline in Calgary, AB, which has recently ended after enrolling 52 patients (ClinicalTrials.gov Identifier: NCT00559494).

Simvastatin (Zocor®) is one of many hydroxymethylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitors that are widely used clinically as cholesterol-lowering agents. In addition to this, statins possess numerous other anti-inflammatory and anti-apoptotic properties that have interested scientists for their potential neuroprotective effects. Like minocycline, statins too have been studied in models of a variety of neurologic conditions, such as stroke, brain injury, and spinal cord injury (Balduini et al., 2003; Chen et al., 2003; Cui et al., 2009; Holmberg et al., 2006; Holmberg et al., 2008; Lu et al., 2007; Mahmood et al., 2008; Mahmood et al., 2009; Ohsawa et al., 2008; Sironi et al., 2003; Wang et al., 2007; Wu et al., 2008; Wu et al., 2009; Zacco et al., 2003). Atorvastatin has been reported to reduce secondary damage and improve behavioral recovery in animal models of thoracic SCI (Dery et al., 2009; Pannu et al., 2005; Pannu et al., 2007). Simvastatin, a more lipophilic statin than atorvastatin, promotes neurite outgrowth in vitro in an inhibitory environment, decreases acute astrocyte activation, and reduces CSPG levels (Holmberg et al., 2006; Holmberg et al., 2008; Ohsawa et al., 2008; Zacco et al., 2003).

Despite these promising reports for minocycline and HMG-CoA inhibitors such as atorvastatin and simvastatin, other investigators have not seen significant improvements in contusion models of SCI (Diguet et al., 2004; Tsuji et al., 2004; Yang et al., 2003). Holmberg et al. (2008) observed no functional recovery in animals treated with simvastatin after an acute SCI, although there were some subtle histopathologic differences reported. In a NIH-funded replication study of minocycline, a significant reduction in secondary damage and improvements in functional recovery were not demonstrated (Pinzon et al., 2008). Recently, we reported on a series of negative studies using simvastatin in thoracic SCI, where repeated attempts to replicate a promising trend from a small pilot study were all met conclusively with no sign of neurologic efficacy (Mann et al., 2010).

In this study, I hypothesize that both minocycline and simvastatin will improve neurological and histological outcomes in a contusion model of cervical spinal cord injury. While most preclinical testing of potential treatments is done in thoracic SCI models, the majority of patients suffer spinal cord injuries in the cervical region. We considered the possibility that our failure to observe any significant neuroprotective effects with simvastatin was in some way related to our
thoracic contusion injury itself – either the contusion injury was too biomechanically severe, or the resultant neuropathology was simply too advanced for small biological improvements induced by simvastatin to be manifested in functional recovery. Hence, we felt that it was warranted to test it in a cervical contusion SCI model, where the injury is unilateral and the resultant tissue damage was much less severe. Additionally, we felt that the testing of minocycline in such a model was warranted given that it had not previously been evaluated in a cervical model.
3.2 Materials and Methods

All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Block randomization of animals into different treatment groups occurred after their injuries and was performed using a computer generated randomization code. All analysis of behavioral and histological outcome measurements were performed by a person blinded to the treatment group that the animals were randomized to, and the randomization code was broken only after the data had all been accrued.

3.2.1 Surgical Procedure

**Cervical Spinal Cord Injury Model.** Forty-three Sprague Dawley rats (Charles River Breeding Laboratories) weighing between 300 and 350 grams were anesthetized with 2% isoflurane in oxygen (1 L/min). A subcutaneous injection of buprenorphine (0.02 mg/kg, Temgesic®, Reckitt Benkiser Healthcare Ltd., UK) and 10 mL saline was administered just prior to and 1 and 2 days after the surgery. A dorsal midline incision was made to expose the C4-C6 vertebrae. A C5 hemilaminectomy was performed on the left side, to allow the passage of an impactor tip with a 1.5 mm diameter. The animal was mounted within a clamping system that was developed to firmly grasp and stabilize the spinal column from C4 to C6 (Choo et al., 2009). It was then positioned under the Infinite Horizon impactor (Systems and Instrumentation, Lexington, KY) at a 22.5° angle, so that the impactor tip would travel lateral-to-medial at a 22.5° angle to the vertical midline axis (Figure 3.1). The tip was gently lowered down until it was 4 mm above the dura. The impactor was then triggered to deliver a force of 150 kdyne at 100 mm/s. The device immediately provides the force and displacement delivered during the impact and animals were excluded from further study if the measured peak force exceeded 165 kdyne (i.e. greater than 10% over the intended force of 150 kdyne). Temperature was monitored with a rectal thermometer (TCAT-2LV Temperature Controller, Kopf, Tujunga, CA) during the operation and an incubator was used to maintain a body temperature of 37°C prior to and after the surgery until the animals were fully awake and mobilizing.
**Anterograde Tracing.** To evaluate whether the treatments had encouraged axonal sprouting, we performed anterograde tracing of the corticospinal and rubrospinal tracts. Six weeks after the contusion injury, animals were anesthetized again with an intramuscular injection of ketamine hydrochloride (72 mg/kg; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (9 mg/kg; Bayer Inc., Etobicoke, ON) diluted in 20 mM Phosphate Buffered Solution (PBS) and placed on a stereotaxic frame. An incision was made on the dorsal aspect of the skull, and on the side contralateral to the cervical spinal cord injury, a 5 mm wide by 15 mm long rectangular window was created using a high-speed dental burr. For the corticospinal tract, 0.4 µL of 10% Fluoro-emerald (Molecular Probes, OR) (0.05 µl/30 sec) was manually injected into the sensorimotor cortex at 4 different sites with a Hamilton syringe at a 30° angle and a depth of 3.0 mm. The coordinates for the 4 injections (in millimeters relative to Bregma) were based upon the atlas of Paxinos and Watson: 1. Anterior posterior (AP): +0.3 mm and medial lateral (ML): 1.5 mm; 2. AP: +0.3 mm, ML: 2.5 mm; 3. AP: -0.7 mm, ML: 1.5 mm; and 4. AP: -0.7 mm, ML: 2.5 mm. For the rubrospinal tract, a hole with a diameter of 3 mm was made at the following coordinates relative to Bregma: AP: -6.0 mm, and ML: 0.75 mm. Through this hole, a Hamilton syringe NanoFil sub-microliter injection pump (World Precision Instruments, Saratoga, FL) was inserted to a depth of 7.0 mm perpendicular to the cortex surface, and 0.6 µL of 10% biotinylated dextrane amine (BDA) (Molecular Probes, OR) was injected into the vicinity of red nucleus for 30 mins, as described previously by Bretzner et al. (Bretzner et al., 2008).

**Necropsy.** 1 week after the anterograde tracing (7 weeks post-injury), animals were sacrificed with an overdose of Sodium Pentobarbital (107 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and transcardially perfused with 150 mL of 1x PBS followed by 300 mL of 4% paraformaldehyde (in PB buffer). Five centimeters of the cervical spinal cord centered around the injury site was collected, post-fixed overnight in 4% paraformaldehyde, and cryoprotected in 12%, 18% and 24% sucrose (in PBS). The spinal cords were cut in cross-section at 20 µm thickness and stored at -80 °C.

**3.2.2 Treatment Administration.**

Minocycline hydrochloride (Sigma, Oakville, ON) was dissolved in sterile saline and gently heated until it was completely dissolved, in accordance with the manufacturer’s instructions.
Simvastatin (Merck, Whitehouse Station, NJ) was dissolved in 0.1 M NaOH and 100% EtOH (simvastatin vehicle (SV)) and was incubated at 50°C for 2 hours (Kugi et al., 2002).

One hour after the injury, animals were randomized to the following treatment groups. Because of the temporal differences in the treatment regimes, all animals were “treated” with either drug or vehicle solution for a total of 42 days (Figure 3.2):

1) Minocycline (Mino) (n=9): Intraperitoneal (IP) injections of Mino (90 mg/kg/day) for 3 days followed by saline for 39 days and subcutaneous (SC) injections of SV for 42 days;
2) Simvastatin for 7 days (Sim7) (n=10): IP injections of saline for 42 days and SC injection of simvastatin: 20 mg/kg/day for 3 days and 5 mg/kg/day for 4 days followed by SV for 35 days;
3) Simvastatin for 42 days (Sim42) (n=10): IP injections of saline for 42 days and SC injection of simvastatin: 20 mg/kg/day for 3 days and 5 mg/kg/day for 39 days;
4) Control group (n=10): IP injections of saline (0.7 mL/day) and SC injections of SV (0.35 mL/day) for 42 days. We did not observe any side effects or behavioral impairment due to the injections.

3.2.3 Behavioral Outcome Measures

**Horizontal Ladder test.** The horizontal ladder test was performed 6 days prior to spinal cord injury and at 14 days, 28 days and 41 days after injury. Animals were placed in the horizontal ladder apparatus (Soblosky et al., 2001) and five runs across the ladder were recorded using a high definition video camera (Sony HDR-SR12). The footage of each rat was later analyzed in slow motion and the number steps and numbers of slips for both forelimbs were counted. The number of slips for the left forelimb (ipsilateral to the SCI) was represented as the “percentage of ipsilateral forelimb errors”, calculated by dividing the number of forelimb slips by the total number of forelimb steps. [% ipsilateral forelimb errors = (ipsilateral forelimb errors / total number of ipsilateral forelimb steps) x 100]

**Cylinder Rearing Test.** The cylinder rearing test was conducted 7 days before SCI and at 13 days, 27 days and 40 days after injury. Animals were placed into a clear topless cylinder measuring 30 cm in height and 30cm in diameter (Schallert et al., 2000). As they vertically reached up with their forelimbs to explore the cylinder wall, 20 rears were recorded using a high definition video camera (Sony HDR-SR12). The footage was later analyzed in slow motion and the number of times the
animal reached up and touched the wall with its left forelimb, right forelimb, or both forelimbs was counted. We considered that the animal was using its “injured” ipsilateral forelimb when it reached up and touched with cylinder wall with its left paw alone, or when it did so with both left and right paws simultaneously. The frequency of the injured forelimb usage was represented as the “percentage of ipsilateral forelimb usage”, calculated by adding the number of times the animal reached up and explored with either its left paw alone or with both paws simultaneously, and dividing this by the total number of paw placements. [% ipsilateral forelimb usage = (left forelimb only + left and right forelimb simultaneously) / total forelimb usage x 100]

Modified Montoya’s Staircase. The modified Montoya’s staircase test was conducted 5 days prior to spinal cord injury and at 15 days, 29 days and 42 days after injury. Fine motor control was assessed using the modified Montoya staircase in which animals reach and grasp food pellets contained in wells at 7 differing depths (Montoya et al., 1991; Nikkhah et al., 1998). Animals were first acclimatized to the apparatus and food pellets (Bio-Serv, Frenchtown, NJ) before the injury by placing the apparatus in their home cage for 2 weeks before injury. There were a total of 14 pellets on each side (2 pellets in each well). For each time point that was assessed, the animals were fasted for 20 hours before being placed in the staircase for 15 mins. The number of pellets retrieved from each well was counted afterwards. “percent pellet retrieval” of ipsilateral forelimbs was calculated by dividing the number of pellets retrieved by 14. [% pellet retrieval = (number of pellets retrieved/14) x 100]

Grooming Test. The grooming test was performed 6 days before SCI and at 14 days, 28 days and 41 days after injury. Animals were placed into a clear topless cylinder measuring 30 cm in height and 30 cm in diameter. A few drops of sterile saline were applied between the ears, and their grooming response was recorded for 15 mins using a high definition video camera (Sony HDR-SR12). The footage was later analyzed to assign a grooming score developed by Gensel et al. (2006).

Sensory Testing for Mechanical Allodynia. The sensory test was conducted 4 days prior to SCI and 16 days, 30 days and 43 days after injury. Forelimb sensitivity was measured using the electronic von Frey aesthesiometer (Dynamic Plantar Aesthesiometer, Harvard Apparatus, Hollister, MA). Animals were placed in a clear topless cylinder measuring 30 cm in height and 30 cm in diameter. Once the animal had acclimatized to its setting, the filament was aimed in the center of the palm and triggered to deliver a mechanical force from 0 g up to 50 g. The force at which the animal
withdraws its forelimb from the filament (the “withdrawal force”) was measured 3 times on each forelimb.

### 3.2.4 Histological Outcome Measures

**White Matter and Grey Matter Sparing.** The sections were stained with Eriochrome Cyanine (EC) as described by (Rabchevsky et al., 2001) counter-stained with Neutral Red, then photographed on a Leica DM5000B microscope at 2.5x objective. Regions of intact white and grey matter on both the ipsilateral and contralateral side of the spinal cord were manually traced and then quantified using SigmaScan Pro version 5.0.0 (Systat Software Inc.). To take advantage of the unilateral nature of the injury, the extent of white and grey matter sparing that was quantified on the injured side was expressed as a percentage of the uninjured side of the spinal cord on each section. The “epicenter” was defined as the cross-section with the least amount of white and grey matter sparing.

**Axonal Sprouting.** To amplify the BDA and Fluorogreen signals for assessment of axonal sprouting, sections were washed three times for 5 minutes in 0.01 M PBS and blocked with normal Donkey Serum (1:10, Jackson Immuno Research Laboratories) for 30 minutes. For visualization of the fluoro-emerald traced CST axons, cross-sections from rostral 3200 to caudal 3200 were incubated in Goat anti-Fluorescein (1:200, Invitrogen) diluted in 0.01 M PBS-Triton X-100 (0.1%) and incubated overnight at room temperature. The following day, the sections were washed three times for 5 minutes in 0.01 M PBS and incubated in FITC-conjugated Donkey anti-Goat antibody (1:400, Jackson Immuno Research Laboratories) for the CST. For visualization of the BDA labeled RST axons, sections were incubated with Cy3 conjugated streptavidin for 2 hrs. Slides were washed three times for 5 minutes in 0.01 M PBS and cover-slipped with Fluoromount G (Southern Biothece).

One series of spinal cord sections 400 µm apart were photographed on a Leica DM5000B microscope at 10x objective. From rostral 2400 µm to caudal 2400 µm, axons in the grey matter regions directly adjacent to the CST and RST were hand traced and average intensity was measured using SigmaScan Pro version 5.0.0 (Systat Software Inc.). The intensity values were normalized to the average intensity of the uninjured cross-sections of C2 to control for tracing variations.
3.2.5 Simvastatin and Minocycline Bio-activity

Twenty five Sprague Dawley rats (Charles River Breeding Laboratories) weighing between 300 and 350 grams were anesthetized with 2% isoflurane in oxygen (1 L/min). Animals were randomized into five groups: sham (n=5), minocycline (n=5), injury only (SCI with SV, n=5), simvastatin no injury (n=5), simvastatin injury (SCI with simvastatin, n=5). Baseline blood samples were drawn from the tail vein prior to injury. Minocycline was administered at 90 mg/kg/day for 3 days and a blood sample was collected 2 hours after the last injection to confirm the systemic delivery of minocycline. Simvastatin was administered at 20 mg/kg/day for 3 days and at 5 mg/kg/day for 11 days in rats with or without unilateral cervical spinal cord injury (as described above). Blood samples were drawn 2 hours after the 3rd day and after the final simvastatin dose 2 weeks post-treatment.

To confirm the systemic delivery of minocycline, serum samples were sent to Calgary Laboratory Service (Calgary, AB). To confirm the bio-activity of simvastatin, serum HDL and LDL levels was measured using a HDL and LDL/VLDL quantification kit (Catalog# K613-100, BioVision, Inc., Mountain View, CA).

3.2.6 Statistical Analyses

All behavior assessments and simvastatin and minocycline bioactivity were compared using a two way repeated measures ANOVA test followed by least significant difference (LSD). To discern the differences between the groups at each time point, a one way ANOVA test followed by LSD was used. Histological assessments were compared using a one way ANOVA test followed by LSD. P value less than 0.05 was considered statistically different.
3.3 Results

Forty-three animals underwent cervical C5 unilateral contusion injuries. Four animals were excluded due to peak injury forces that exceeded 165 kdyne. The remaining 39 animals were randomized to receive minocycline (Mino; n=9), simvastatin for 7 days (Sim7; n=10), simvastatin for 42 days (Sim42; n=10) or saline (Control; n=10). The average peak forces (154.7 ± 1.1 vs. 156.4 ± 1.7 vs. 155.5 ± 1.1 vs. 158.3 ± 2.0 kdyne, p=0.39) and impactor tip displacements through the cord (1.52 ± 0.05 vs. 1.55 ± 0.05 vs. 1.48 ± 0.05 vs. 1.58 ± 0.04 mm, p=0.41) between groups were comparable.

3.3.1 Behavioral Outcome Measures

Horizontal Ladder Test. The injury caused significant impairment of forelimb placement at 2, 4 and 6 weeks, compared to baseline pre-injury placement, as illustrated by a substantial increase in ipsilateral forelimb percent errors after injury in all four groups (Control; Mino; Sim7; Sim42). As is typical for incomplete paralysis, some degree of spontaneous recovery did occur, with the ipsilateral forelimb percent error being approximately 30% at 2 weeks, but then recovering to about 20% at 4 and 6 weeks in all groups. Interestingly, neither minocycline nor simvastatin treatment improved ladder performance post injury (Figure 3.3). No significant differences in ipsilateral forelimb errors were observed between any of the groups. At 6 weeks post-injury, the ipsilateral forelimb percent error was Control: 15.49 ± 2.85 %, Mino: 14.85 ± 1.96 %, Sim7: 19.24 ± 2.71 % and Sim42: 17.46 ± 2.79 % (Figure 3.3).

Cylinder Rearing Test. Ipsilateral forelimb usage was dramatically reduced at 2, 4 and 6 weeks post-injury in all groups, but there were no significant differences between the groups (Figure 3.4). At 6 weeks, the percent ipsilateral forelimb usage was Control: 7.14 ± 3.76 %, Mino: 12.45 ± 5.43 %, Sim7: 7.95 ± 2.49 % and Sim42: 12.93 ± 5.53 %.

Grooming Test. Compared to the pre-injury state, a dramatic decline in the grooming score was observed after injury for all the groups (Figure 3.5). However, the grooming score of rats in the Sim42 group was significantly higher compared to Control, Mino and Sim7 at 6 weeks (Sim42: 2.78 ± 0.22, Control: 1.90 ± 0.18, Mino: 2.00 ± 0.22 and Sim7: 2.00 ± 0.32, p=0.003).
were no differences between animals in the Control, Mino and Sim7 group throughout the experiment.

*Modified Montoya’s Staircase Test.* After SCI injury, all rats expectedly demonstrated a marked drop in grasping performance and the number of food pellets retrieved. However, grasping performance was not improved in the Mino, Sim7 and Sim42 groups, as compared to controls. In fact, the Sim7 group performed significantly worse than the Control and Mino groups at week 2 and the Sim42 group performed significantly worse than the Control and Mino groups at week 6 (Figure 3.6, p=0.002). Of note, there were also no differences in weight between the treatment groups that might influence feeding behavior.

*Sensory Testing for Mechanical Allodynia.* At 6 weeks post-injury, there was no difference in the force for ipsilateral forepaw withdrawal between the animals treated with Control, Mino, Sim7 and Sim42 (Figure 3.7). In all groups, the injury itself caused a significant reduction of the ipsilateral forepaw sensitivity, as evidenced by the increased force required before the animal withdrew the forelimb (p<0.05). No suggestion of mechanical allodynia and “hyper-sensitivity” was observed, in which withdrawal of the forelimb occurred with decreased force.

### 3.3.2 Histological Outcome Measures

*White matter and grey matter sparing.* Seven weeks after injury, the gross tissue damage extended 2800 µm rostrally and 2800 µm caudally to the injury site (Figure 3.8). The extent of grey and white matter sparing through this region was measured in serial cross sections 400 microns apart. There were significant differences at c400 and c800 between Mino compared to Sim7 and Control for white matter sparing (p<0.0001), but there were no significant differences between the groups for grey matter sparing. The cumulative extent of white matter sparing or of grey matter sparing did not differ significantly between the Control, Mino, Sim7, and Sim42 groups.

*CST and RST sprouting.* The extent of CST and RST sprouting was quantified from r2400 to c2400 (Figure 3.9). At 7 weeks post injury, there were no statistically significant differences between the Control, Mino, Sim7 and Sim42 groups.
3.3.3 Simvastatin and Minocycline Bio-activity

Plasma minocycline levels two hours after the 3rd dose were, on average, 40.34 ± 6.70 mg/l. This level was generally higher compared to previously reported plasma minocycline levels (32.1 – 34.7 mg/l) that was administered IP at a dose of 90 mg/kg initially followed by 45 mg/kg every 8 hours for the first 24 hours and 45 mg/kg/day for two additional days (Fagan et al., 2004).

For simvastatin bio-activity (Figure 3.10), in both the sham animals and SCI animals, treatment with simvastatin led to significantly reduced HDL/LDL ratios at 3 days and 2 weeks compared to the sham and SCI animals not treated with simvastatin (p<0.001 and p=0.003).
3.4 Discussion

In this study, we utilized a clinically relevant unilateral contusion spinal cord injury model to investigate the neuroprotective potential of minocycline or simvastatin, the latter of which was evaluated with treatment regimens extending over 7 days or over 42 days. Histologically, there were no significant increases in white matter sparing, grey matter sparing or axonal sprouting for either minocycline or simvastatin. Functionally, there were no significant improvements in the horizontal ladder test, cylinder rearing test, or modified Montoya staircase. In the case of the modified Montoya staircase, which evaluates forelimb reaching, the simvastatin treatment may have even slightly worsened the animals’ performance. The only “improvement” we observed was in the grooming test for those animals receiving simvastatin over 42 days. We believe these behavioral observations – where the animals groomed more vigorously in their response to the drop of saline on the back of their heads and animals were discouraged to enter a narrow space resulting in less pellet retrieval in the staircase- were actually related to the local irritation of the subcutaneous tissues caused by the simvastatin, and not to a bona fide neurologic improvement. Such local irritation and inflammation was documented previously in a study where simvastatin was injected into the subcuticular region of the dorsal thorax (Joles et al., 1992).

A unilateral contusion injury of the cervical spinal cord has a number of translational considerations. Firstly the majority of acute spinal cord injuries occur in the cervical region (NSCISC, 2009), and because of the difficulty in measuring local, segmental motor recovery in thoracic SCI, future clinical trials of acute neuroprotective drugs may exclude thoracic SCI patients entirely and focus exclusively on cervical SCI patients. Hence, there is a strong rationale to study potential neuroprotective agents in a model of cervical SCI, and to study forelimb function as a measure of local recovery. In terms of the mechanism of injury, the contusion SCI attempts to reproduce the dynamics of the injury, and is considered by the majority of the research field to be the most clinically relevant mechanism of non-penetrating injury (Kwon et al., 2010a). An additional advantage of the cervical unilateral contusion SCI model is the battery of forelimb behavioral tests that are available to distinguish different aspects of forelimb function. In this study, we utilized the horizontal ladder test, cylinder rearing test and grooming test for “gross” forelimb movements and the modified Montoya staircase for “fine” grasping movements. While locomotion after thoracic SCI is often attributed to intrinsic central pattern generators functioning without
supraspinal input, such a confounder is considered to be less of an issue after cervical SCI, where skilled forelimb function is considered to be primarily a reflection of descending tracts and local/segmental neuronal circuitry. The fact that the cervical injury is unilateral and the gross damage is restricted to the one side of the spinal cord allows for histological comparison with the contralateral side of the spinal cord. This allows each animal to serve as an internal control when quantifying grey and white matter sparing. Finally, from a practical perspective, post-injury morbidity is low with such a unilateral injury. The animals display little pain behavior and manual bladder expression is not necessary.

In the cervical unilateral contusion model that was utilized in this study, the animal’s functional deficits were restricted to the injured side throughout the experiment. Our injury appears to be more severe both behaviorally and histologically compared to other cervical injury models that have employed laceration, compression, or contusion injury mechanisms. These injury models report forelimb impairment on the horizontal ladder test, with error levels in the range of 5 to 10%, in contrast to our study with an error rate closer to 20% even after 6 weeks (Gensel et al., 2006; Girgis et al., 2007; Plunet et al., 2008; Soblosky et al., 2001). For the Cylinder Rearing test, the deficits in ipsilateral forelimb usage were about 10%. This deficit is comparable to that observed by (Soblosky et al., 2001) using a weight drop (10.5 grams) at 5 cm and also similar to that observed by Gensel et al. who utilized a New York University (Basso et al., 1996) impactor with a 12.5 mm drop height (Gensel et al., 2006).

In the grooming test, the extent of impairment was similar in the early time points after the injury when compared to the 12.5 mm NYU impactor injury by Gensel et al.; however, by 6 weeks, our animals continue to be quite impaired (score: 2) compared to the grooming score of 3 in the study by (Gensel et al., 2006). Surprisingly, there are no other cervical SCI studies that have employed the modified Montoya Staircase test of food pellet reaching. We contend that this is a relatively easy method for assessing a fine-motor function of the forelimb. Histologically, both the white and grey matter on the injured side are severely damaged at the lesion epicenter, with virtually no grey matter spared at the epicenter. The extent of spared white matter in our injury model is comparable to what we have previously seen using the OSU impactor and a 1.5 mm contusion injury (Lee et al., 2010).

Statins such as simvastatin (Zocor®) are known to have pleiotrophic effects beyond their recognized lipid-lowering activity. By blocking the synthesis of mevalonate and downstream
isoprenoid derivatives, statins can influence inflammation and RhoA activation, both of which are implicated in the secondary injury cascade after acute neurotrauma (Dergham et al., 2002; Erschbamer et al., 2005; Holmberg et al., 2008; Madura et al., 2004). Simvastatin has been extensively studied in ischemic and traumatic brain injury (Mahmood et al., 2008). In culture, simvastatin is shown to be neuroprotective by suppressing apoptosis induced by NMDA receptor mediated excitotoxicity (Zacco et al., 2003). It is demonstrated to decrease astrogliosis, have anti-inflammatory effects by reducing IL-1ß, IL-6 and TNF-α leading to reduced secondary damage, induce BDNF expression, promote neuronal survival and out-growth, reduce apoptosis, and improve functional outcomes in TBI (Lu et al., 2007; Mahmood et al., 2008; Mahmood et al., 2009; Wang et al., 2007; Wu et al., 2008; Wu et al., 2009). In stroke models, simvastatin improves angiogenesis, arteriogenesis, improves functional recovery, reduces lesion volume and inflammation by decreasing IL-1ß and TNF-α (Balduini et al., 2003; Chen et al., 2003; Cui et al., 2009; Sironi et al., 2003). In SCI, simvastatin is also reported to promote neurite outgrowth in an inhibitory environment, decrease acute astrocyte activation, reduce CSPG levels and lessen nociceptive response (Holmberg et al., 2006; Holmberg et al., 2008; Ohsawa et al., 2008).

Despite this past literature on the neuroprotective properties of simvastatin, we found that it did not reduce secondary injury or improve function in a cervical unilateral contusion SCI model, results which echo those that we observed in a thoracic contusion injury model and reported previously (Mann et al., 2010). We undertook this current cervical SCI study in part to address the question of whether the lack of a beneficial effect in our thoracic SCI paradigm might be related to the thoracic injury model itself. We have used, after all, a simvastatin dose and mode of administration that others had experienced success with in models of neurologic disorders (Balduini et al., 2001; Balduini et al., 2003; Endres et al., 1998; McGirt et al., 2002; Sironi et al., 2003; Wang et al., 2007). We considered, for example, that perhaps the lack of improvement on the BBB after simvastatin treatment for our thoracic cord injury was due to the biomechanical severity of the thoracic injury, and a “ceiling effect” on the locomotor recovery (Mann et al., 2010). In that study, we observed a quite significant cavitation through the epicenter of injury, and postulated that perhaps in a unilateral cervical model, where there would definitely be more tissue sparing and segmental cord recovery could be measured with forelimb use, a beneficial effect might be observed. As our results show, neither the short term (7 days) or long term (42 days) simvastatin treatment improved the outcome in our cervical SCI model.
The doses and treatment duration of simvastatin were similar to that which we utilized previously (Mann et al., 2010) and these were carefully selected based on previous studies which reporting therapeutic effects. Many of the studies that have investigated simvastatin in models of neurologic disorders utilized an oral treatment in the range of 0.57 to 2.3 mg/kg/day (Abrahamson et al., 2009; Holmberg et al., 2008; Li et al., 2009; Mahmood et al., 2008; Mahmood et al., 2009; Wu et al., 2008; Wu et al., 2009). There are also studies demonstrating the beneficial effects of intraperitoneal and subcutaneous administration of simvastatin at 20 mg/kg (Balduini et al., 2003; Ohsawa et al., 2008; Sironi et al., 2003). A pilot study that we conducted previously to ensure the tolerability of this dose revealed that the animals showed signs of systemic toxicity after 3 days of 20 mg/kg/day, and so we decided to lower the dose to 5 mg/kg/day for the remainder of the treatment. We elected to employ a simvastatin dose of 20 mg/kg and 5 mg/kg, as these doses and mode of administration has been previously reported to be beneficial in stroke models (Balduini et al., 2001; Balduini et al., 2003; Endres et al., 1998; Lu et al., 2007; McGirt et al., 2002; Sironi et al., 2003; Wang et al., 2007). While we assumed that tissue neuroprotection would be achieved with early intervention, we also employed an extended, 42-day treatment regimen to test the hypothesis that the prolonged application of simvastatin might also promote axonal sprouting, as suggested by its influence on the Rho pathway (Holmberg et al., 2008; Pannu et al., 2007).

Of course, with simvastatin not having any therapeutic effect, we did consider whether the drug was actually getting into the systemic circulation and having any biological effect. It should be emphasized that we did select a dose and a mode of administration that others investigators have found to be effective in models of neurologic disorders (Abrahamson et al., 2009; Balduini et al., 2001; Balduini et al., 2003; Endres et al., 1998; Holmberg et al., 2008; Li et al., 2009; Lu et al., 2007; Mahmood et al., 2008; Mahmood et al., 2009; McGirt et al., 2002; Sironi et al., 2003; Wang et al., 2007; Wu et al., 2008; Wu et al., 2009). Nonetheless, we did test for the metabolic effects of simvastatin by measuring serum HDL/LDL ratio changes. While most studies that investigate simvastatin for its lipid-lowering effects utilize hyper-cholesterolemic animals (Felgines et al., 1994; Joles et al., 1992; Kasim et al., 1992; Lutton, 1999), in normal SD rats, total cholesterol levels are not affected by simvastatin (Joles et al., 1992; Sironi et al., 2003). While we also did not see changes in total cholesterol levels, we did see a lower HDL/LDL ratio changes in the simvastatin treatments groups. This is surprising, since the HDL/LDL ratio changes are expected to increase with simvastatin treatment. Regardless, the persistent significant differences
between the simvastatin treated and non-simvastatin treated groups indicate that simvastatin was having a systemic metabolic effect on the animals. To our knowledge, there are no other studies that report a decrease in HDL/LDL ratio changes in normal SD rats.

Recently, improved behavioral recovery with simvastatin administration in an *in vivo* rodent thoracic SCI model was reported (Shunmugavel et al., 2010). In this study, a controlled contusion injury was performed at T9/10 and 5 mg/kg of simvastatin was administered via oral gavage 2 hours later, with doses repeated daily for the subsequent 28 days. The authors focus this paper on improved bladder and renal function with simvastatin treatment; while improved BBB scores were observed, this improvement in locomotor scores is only briefly mentioned, no figure is shown of these results, and no histology of the spinal cord was reported to demonstrate a neuroprotective effect. Our contusion injury models are different, and our simvastatin regimen began with a higher dose (20 mg/kg/day) which was reduced after 3 days to 5 mg/kg/day. Additionally, we utilized a subcutaneous route of administration instead of oral gavage. It is difficult to explain the obvious differences between the Shunmugavel et al. paper and our results, not just in the cervical hemicontusion injury but also in the series of thoracic SCI experiments that we previously published on (Mann et al., 2010).

Holmberg et al. (2008) studied simvastatin in a thoracic SCI model using the NYU weight drop model and observed no behavioral improvements, although they did see reductions in microglial activation and GFAP expression. Previous work on statins in SCI models has focused on atorvastatin (Lipitor®), which is less lipophilic than simvastatin and hence has a theoretical disadvantage with respect to crossing the blood brain barrier (Zacco et al., 2003). Nonetheless, atorvastatin has been reported to cause remarkable functional recovery after thoracic SCI by Pannu et al. (2005, 2007), and this efficacy was independently corroborated to some extent more recently by Dery et al. (2009). Our work differs from Pannu et al. in our use of a cervical SCI model (versus thoracic SCI), our use of the force-controlled Infinite Horizon impactor, and our mode of subcutaneous administration (versus oral gavage), although prolonged treatment regimens were employed for both simvastatin and atorvastatin. Our lack of therapeutic effect for simvastatin in this cervical SCI model is similar to that which we observed using the Ohio State University impactor (1.5 mm displacement) at T9/10 (Mann et al., 2010).

Unlike simvastatin, minocycline has already been fairly extensively studied in a spinal cord injury model. Minocycline decreases apoptosis via inhibiting proinflammatory cytokines such as
TNF-α, glutamate toxicity, cytochrome c release from mitochondria and Caspase -1,3 activities (Lee et al., 2003; Teng et al., 2004; Yrjanheikki et al., 1999; Zhu et al., 2002). In addition to its role in cell death, minocycline has also been reported to provide neuroprotection by inhibiting the p38 mitogen-activated protein kinase pathway in microglial/macrophage cells, thereby inhibiting the interaction between excitotoxicity and inflammation (Piao et al., 2003; Tikka and Koistinaho, 2001; Yune et al., 2007). These molecular mechanisms are supported by improved functional outcomes evident by improvements in BBB and reduction of neuropathic pain (Marchand et al., 2009; Yune et al., 2007).

For minocycline, the dosage of 90 mg/kg/day for 3 days was based on 1. the study by Stirling et al. (2004) that reported neuroprotective effects of minocycline at 100 mg/kg/day administered IP in a dorsal hemisection cervical SCI, 2. various studies reporting beneficial effects with IP administration of minocycline at 90 mg/kg/day (Festoff et al., 2006; Lee et al., 2003; Teng et al., 2004; Yune et al., 2007) and 3. from previous work in our laboratory examining that revealed modest effects with IV administration of minocycline at 90 mg/kg/day in a thoracic contusion model (unpublished results).

Our results with the use of minocycline add to the growing body of preclinical literature on this drug in acute SCI. A systematic review of the literature up until June 2009 revealed 10 studies of minocycline, 8 of which reported on beneficial effects, and 2 of which reported no effect (not including our current study) (Kwon et al., 2010b). One such negative study was a formal NIH-funded replication study, which failed to reveal any histological or functional improvements after minocycline treatment (Pinzon et al., 2008). It is difficult to precisely identify the cause of these very different results. It is recognized that there are difficulties replicating positive experimental results due to differences in animal species and strain, weight, injury model, injury severity, and outcome assessors. However, if a given treatment’s effect is not sufficiently robust to withstand these experimental variations within the controlled preclinical environment, the likelihood of it demonstrating efficacy within the wide variability of human spinal cord injury is probably quite small. We acknowledge that there may be factors related to our injury model and treatment paradigm that influenced the outcome. We considered the possibility that the injury itself is too severe for neuroprotection to occur; recent work in our lab in which the same model has been used for the assessment of a ketogenic diet suggests that there is indeed the potential for a treatment to induce improved behavioral and histological outcomes (W. Tetzlaff, unpublished results).
conceivable that the bio-availability of the drug at the injury site was insufficient to effectively attenuate secondary damage mechanism, either due to dosing issues or to the disruption of local vasculature. Or perhaps the drugs were indeed working while they were being administered early after injury, but after being stopped, the secondary damage proceeded in an unmitigated fashion, leading to the same result in the end. Alternatively, it is possible that the time delay we used (1 hour post-injury) was too late, and that the treatment needs to be administered even earlier to be effective (although if this were the case the treatment would have limited clinical applicability). All of these specific factors may be considered, although we also recognize that one cannot be so “choosy” with the spinal cord injuries that human patients arrive with, and so to be found clinically effective across the spectrum of injuries, a treatment must be able to overcome some of these issues.

Our results raise interesting questions about the translational potential of simvastatin and minocycline for human SCI. In the case of simvastatin, we previously conducted a fairly extensive series of experiments in a thoracic SCI model to eventually (and somewhat painfully) come to the conclusion that simvastatin was not providing any neuroprotective benefit (Mann et al., 2010). We now have demonstrated that neither a short term (7 day) or long term (42 day) simvastatin treatment regimen provide any benefit in a cervical SCI model. Holmberg et al. (2008) are the only other group to have previously reported on simvastatin in an acute thoracic SCI model, and they too found no behavioral benefit (albeit with a much lower dose than we applied). Based on the available preclinical literature on simvastatin for acute SCI, it would seem that there is little to substantiate it as a potential neuroprotective agent for SCI, outside of the theoretical advantages it might have over atorvastatin, which has been found to be beneficial in models of thoracic SCI (Dery et al., 2009; Pannu et al., 2005; Pannu et al., 2007).

Minocycline, on the other hand, represents a more complex issue. As preclinical data accumulates on minocycline in acute SCI models, uncertainty arises about how to interpret such negative data in the face of other well-conducted studies which report positive results. The scientific community clearly acknowledges the existence of a publication bias towards positive data and the reluctance of investigators to publish negative data (Kwon et al., 2010a). How to then “weigh” such published negative data in the considerations of minocycline’s “promise” for human SCI is unclear. A poll of scientific experts from a recent focus group meeting on SCI translational research suggested that such negative studies be considered to be as important as positive studies in assessing a potential therapy’s promise and readiness for human translation (Kwon et al., 2010b).
Of course, for minocycline, this is complicated by the fact that it has already been translated into humans, with a pilot study of acute SCI patients now being completed in Calgary, Alberta. At the time that this clinical trial was initiated in 2004/2005, there were multiple independent laboratories that had published on the beneficial effects of minocycline after acute SCI, making it arguably the most promising pharmacologic agent for human translation available at that time. How the results of subsequently published negative preclinical studies should be considered in the decision to proceed with a larger multi-center clinical trial of minocycline for acute is unclear. Arguably, the clinical data itself from the pilot study is of greatest significance, but - like most small clinical trials – strong conclusions about efficacy that might convince one to proceed or to not proceed with a larger study are typically quite elusive. A painful reminder of this fact is the exciting and promising results for GM-1 ganglioside that were reported in a small, 37 patient single-center clinical trial of acute SCI in 1990 (Geisler et al., 1991). This served as the stimulus for a large, 760-patient multicenter clinical trial conducted by over 25 neurotrauma institutions across North America, which unfortunately was negative (Geisler et al., 2001). So, if even clinical results from small and preliminary human trials cannot assure future success in larger multi-center randomized trials, how should one view the ongoing publication of positive or negative preclinical results, such as ours? This remains the matter of some uncertainty and interpretation, and we would not propose to have a definitive answer to this issue. Perhaps, if anything, such negative data injects caution into the research community and brings the expectations down to more realistic levels about the extent of neuroprotection that such drugs may confer to human patients.
Figure 3.1  A. Infinite Horizon Impactor.  B. Top view and width of the frame (box from A.).  C. Front view and height of frame.  D. Side view of the frame and 22.5° angle.  E. Blown up image of the clamp (Choo et al., 2009) (box from C.).  F. Cross section of a spinal cord with the impact trajectory angle.
**Figure 3.2  Schematic of Treatment Groups**

<table>
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<tr>
<th>Week 1</th>
<th>Week 2</th>
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<tr>
<td>Minocycline (Mino)</td>
<td>Minocycline 90 mg/kg/day IP</td>
<td>Saline IP</td>
<td>Simvastatin Vehicle SC</td>
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<tr>
<td>Simvastatin 7 days (Sim7)</td>
<td>Simvastatin 20 mg/kg/d SC</td>
<td>Simvastatin 5 mg/kg/d SC</td>
<td>Simvastatin Vehicle SC</td>
<td>Saline IP</td>
<td></td>
</tr>
<tr>
<td>Simvastatin 42 days (Sim42)</td>
<td>Simvastatin 20 mg/kg/d SC</td>
<td>Simvastatin 5 mg/kg/d SC</td>
<td>Saline IP</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>Simvastatin Vehicle SC</td>
<td>Saline IP</td>
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Figure 3.3 **Horizontal Ladder Test.** Left. Ipsilateral percent error: there are no significant difference between any of the groups. Right. Top: Image of a rat traversing across the irregular rungs. Bottom: Image of a rat making an error. (Percent error = (number of ipsilateral slips/total ipsilateral steps) x 100)
Figure 3.4 Cylinder Rearing Test. Left: Ipsilateral percent usage. There are no statistical
difference between any of the groups. Right: Image of an animal exploring the cylinder.
(Percent use = \(\frac{(\text{number of ipsilateral rears} + \text{number of both rears})}{\text{total number of rears}} \times 100\))
Figure 3.5 Grooming Test. Left: Ipsilateral grooming score. Sim42 group scored significantly higher compared to the Control group at 2 and 4 weeks (p=0.018 and p=0.045). Sim42 group scored significantly higher compared to Control, Mino and Sim7 at 6 weeks after injury (p=0.022).

Right: Grooming score description.
Figure 3.6 Modified Montoya’s Staircase Test. Left: Number of pellets retrieved on the ipsilateral side. Sim7 collected significantly lower number of pellets at 2 weeks and Sim42 retrieved significantly less number of pellets at 6 weeks compared to the Control and Mino groups (p=0.047 and p=0.025). Right: Image of an animal retrieving pellets from the wells.
**Figure 3.7 Sensory Test.** Ipsilateral forelimb withdrawal force. Sim7 group withdrew at a lower force compared to the Mino group at 2 weeks.
Figure 3.8 White and Grey Matter Sparing. A. Representative images of injury pattern from rostral 1600 µm to caudal 1600 µm. B. White matter and grey matter sparing. There is a statistical difference between Mino and Sim7 at c400 and c800 (p=0.001 and p=0.01). C. Cumulative white matter and grey matter sparing. There are no statistical differences between any of the groups for grey matter, cumulative white matter and cumulative grey matter sparing.
Figure 3.9  Corticospinal and Rubrospinal Tract Tracing  

A. Corticospinal Tract Tracing.  Left: Representative image of a traced corticospinal tract (2.5x).  Right: Area of corticospinal tract sprouting analysis (10x) (inset box from left).  B. Left: Representative image of a traced rubrospinal tract (2.5x).  Right: Area of rubrospinal tract sprouting analysis (10x) (inset box from left).  C. Corticospinal and rubrospinal tract analysis: There were no differences between any of the groups.
Figure 3.10 Simvastatin Bioactivity. At 3 days and 2 weeks, both simvastatin with or without injury had lower ratio change compared to sham and injury only group (p<0.0001).
3.5 References


CHAPTER 4

Conclusion
4.1 Objectives

Through series of experiments, I developed a clinically relevant cervical unilateral contusion model and used it to test two neuroprotective agents for spinal cord injury. The impactor employed to develop the injury model was the Infinite Horizon Spinal Cord Injury (IH) device. The force-controlled nature and ease of use have recently increased the popularity of this impactor for thoracic injury models. As most injuries occur in the cervical level in humans, I refined the biomechanical, functional and histological parameters of the IH impactor to develop a cervical unilateral contusion model.

Minocycline and simvastatin have been extensively tested in various CNS disorders and have been shown by some investigators to improve neurological outcomes (Cui et al., 2009; Mahmood et al., 2009; Stirling et al., 2004; Wells et al., 2003). In addition, as they are already in clinical use for other indications, their safety and tolerability are well established. In experimental SCI, while some investigators have reported both positive results for these agents (particularly minocycline), others have reported the lack of any beneficial effects. We and others have previously tested minocycline and simvastatin in a thoracic contusion model and were unable to demonstrate the therapeutic effects (Mann et al., 2010). As the existence of the central pattern generator and ceiling effects makes interpretation of thoracic injury models difficult, I have considered the possibility that the nature of the thoracic injury model itself may have contributed to the lack of treatment effects. In addition, to date, these two drugs have not been tested in a cervical contusion model.

The overall objectives of my thesis were to:

1. Develop a clinically relevant cervical unilateral contusion model using the Infinite Horizon Spinal Cord Injury device.

2. Determine if minocycline or simvastatin improved neurological recovery in this cervical injury model.
4.2 Recap of Results

4.2.1 Development of Cervical Unilateral Contusion Model Using the IH Injury Device

As we had prior experience developing a unilateral contusion model using the Ohio State University impactor my initial experimental approach was similar: set up the animals in a neutral fashion (0° rotation) and injure them at three different forces (75, 100 and 150 kdyne) (Lee et al., 2010). Using the Infinite Horizon Impactor, an abnormal injury occurred where a force spike appeared on the force vs. time graph during the injury. Functionally, the animals with the force spikes recovered to baseline levels by 4 weeks, as did those with normal 75 kdyne injuries (without the force spikes). There was a sustained functional deficit with the 100 kdyne injuries, but the impairment was so mild that it would have been impossible to “promote recovery” with a therapeutic intervention. Hence, we decided that the force should be set at minimum 150 kdyne. In the 2nd experiment, to reduce the occurrence of abnormal force spikes, animals were rotated at 17.5°, 20.0°, 22.5° and 25.0° off the vertical axis. Functional analysis revealed that animals rotated at the 22.5° and 25.0° produced sustained and significant deficits on the horizontal ladder test, cylinder rearing test and staircase test. However, histological analysis revealed that some the damage crossed the midline to the contralateral side. Finally, to locate the proper lateral aim to minimize the extent to which the injury crossed to the opposite side, three different lateral aims were tested at 1.0, 1.2 and 1.4 mm off the midline. As expected, 1.4 mm aim off the midline resulted in the fewest number of animals with injury expanding to the other side. Through these series of experiments, I have established a novel cervical unilateral contusion model with the IH impactor, using the parameters of 150 kdyne force, 22.5° (or 25.0°) angle and 1.4 mm lateral aim.

4.2.2 The Effect of Minocycline, Short-term and Long-term Simvastatin Treatment Using a Cervical Unilateral Contusion Model

Minocycline and simvastatin treatment did not display neuroprotective properties nor did they promote axonal sprouting after contusive cervical SCI. As mentioned, previously, we have reported that minocycline and simvastatin do not display neuroprotective effects after thoracic SCI. Therefore we evaluated these two drugs in a cervical unilateral contusion model that was developed
in the first part of this thesis in the hope that we would be able to tease out and demonstrate subtle
treatments effects if they existed. Minocycline was delivered intraperitoneally and simvastatin was
administered subcutaneously. Both routes have been previously described as being effective modes
of administration for these specific drugs in various CNS diseases. We additionally confirmed the
delivery of these drugs in our hands by measuring serum minocycline levels and HDL/LDL, as
simvastatin is well known to increase HDL and decrease LDL. Functional recovery was assessed
using the horizontal ladder test, cylinder rearing test, grooming test and modified Montoya staircase
test. Forelimb sensitivity was measured using the electronic von Frey aesthesiometer. Histological
analysis was examined by spared white and grey matter, cumulative white and grey matter and the
corticospinal and rubrospinal tract was traced to visualize axonal sprouting. We were not able to
show any functional and histological differences between any of the groups.
4.3 Discussion

The discussions of the results of injury model development and minocycline/ simvastatin is at the end of Chapter 2 and Chapter 3.

Preclinical evaluation of a therapy should ideally be performed using an experimental model that most closely resembles human spinal cord injury; however, it has been stated that no single injury model replicates all aspects of human SCI (Kwon et al., 2002; Onifer et al., 2007). There are various experimental injury mechanisms available to study SCI, for example, contusion, compression, laceration and chemical. It remains difficult to determine which experimental model most accurately portrays the sequence of pathological events that occurs in humans (Kwon et al., 2002; Onifer et al., 2007). Although contusion injuries closely stimulate the molecular and pathophysiological process of human SCI (Gensel et al., 2006; Kwon et al., 2002; Kwon et al., 2010a), typically a combination of contusive and compressive injuries occur in clinical settings.

As the IH impactor is one of the most widely used devices to study SCI, the cervical unilateral contusion model developed in this thesis can be used as a valuable preclinical tool to test various therapies in the cervical level. This injury model is user friendly and reproducible. It is distinct in that there are sufficient functional deficits for treatments to display improvements and it is the only injury model that contains the damage on the ipsilateral side. These features make this injury model a true unilateral cervical contusion model that is available to study SCI. So far, this injury model has been utilized to test minocycline, simvastatin and ketogenic diet (KD) and the lack of neuroprotective effects of minocycline and simvastatin have been demonstrated in Chapter 3 of this thesis. On the other hand, both functional and histological improvements of KD administration after SCI have been captured using the cervical unilateral contusion model developed in this thesis (Steijger et al., manuscript in preparation).

Both minocycline and simvastatin targets multiple secondary mechanisms. Minocycline attenuates apoptosis and inflammation (Ha et al., 2008; Stirling et al., 2004). Simvastatin reduces oxidative stress and promotes axonal sprouting by inhibiting the Rho pathway (Holmberg et al., 2008). Both agents have been tested in experimental SCI models and reported to have conflicting neurological outcomes (Tables 1.3, 1.4). This study was the first to test minocycline and simvastatin in a cervical contusion injury model. Here, I report that administration of minocycline or simvastatin (short term or long term) did not improve functional, histological outcomes after a
cervical SCI. These results corroborate the studies that show the lack or limited therapeutic effects of minocycline and simvastatin after thoracic SCI (Mann et al., 2010; Pinzon et al., 2008).

Having said this, there are some limitations of the injury model. The first limitation is the availability of the clamping system. The reliability and rigidity of the clamping system have already been demonstrated by Choo et al. (2009), however, since the clamping system was custom made, it is not widely available. Second, even though significant effort was made to standardize procedure and produce consistent injuries, an abnormal spike in the force vs. time curve still occurs occasionally (although not frequently), forcing us to eliminate some animals during experiments. Lastly, the contusive mechanism of the injury model makes this arguably the most clinically relevant injury model, but the model does not incorporate sustained compression post-impact. Since combinations of injury mechanisms, in particular, contusive and compressive forces are common in human spinal cord injuries, a compressive mechanism can be added to the contusion model developed in this thesis. The IH impactor not only allows setting the desired force, but it also allows adjusting the dwell time, the duration that the impactor tip is applying the injury. As the purpose of this injury development was to establish a cervical contusion model, experiments with changing the dwell time were not conducted, but to potentially improve upon the clinical relevancy of this injury model, a compressive aspect of the injury can be added on top of the contusion model presented here. To date, it is not clear what role different types of injuries play in injury exacerbation. With this approach, one can determine how much and how long a compressive force contributes to the overall injury that results weeks to months after injury.

It is becoming more and more apparent in preclinical SCI research that beneficial effects of specific pharmacological agents are not always consistently reproduced. Many things may influence these apparently discrepant neurological outcomes: the differences in experimental procedure such as animal strain, animal sex, routes of administration, type of injury and severity of injury (Tables 1.3, 1.4). We considered the possibility that the severity of the injury model may have contributed to the lack of neurologic improvement that we reported for simvastatin and minocycline in a thoracic injury model. However, we saw no benefits in the cervical injury model, despite keeping the drug dose and mode of administration the same. Although the dose and route of administration for minocycline and simvastatin have been selected carefully based on previous positive studies, it is possible that the biological effect of minocycline and simvastatin may have been simply too modest to demonstrated an effect on a cervical unilateral contusion model. While
we did confirm the delivery of both drugs the systemic circulation, but perhaps the bio-availability of the drug at the injury site was insufficient to display a beneficial effect. Lastly, it is possible that even though there were subtle changes in the cord, the sensitivity of the functional outcome measures that were utilized in Chapter 3 of this thesis may not have been sufficient to display it.

For minocycline and simvastatin, this is the third study that was published to show the lack of beneficial effects of minocycline. Despite these studies, there are many more studies that report beneficial effects of minocycline. Minocycline has already been extensively tested in rodents SCI models and now it has just entered phase 2 clinical trials to assess clinical safety and tolerance (ClinicalTrials.gov Identifier: NCT00559494). In phase 1, the therapeutic effects of minocycline were only observed in patients with cervical SCI. However, going through clinical trial is a lengthy process. It took GM-1 ganglioside over a decade to complete phase 2 clinical trials (Geisler et al., 1991; Geisler et al., 2001), so in the mean time, it would be worthwhile to test minocycline in a large animal model to find out if the beneficial effects of minocycline can be demonstrated. Our laboratory is currently developing a porcine SCI contusion model that can be used to test pharmacological agents.

In contrast, simvastatin has not yet been extensively investigated in SCI. To date there is only one study that reports functional recovery after SCI after simvastatin treatment (Shunmugavel et al., 2010). Further studies are necessary to optimize simvastatin dosage and to find an effective route of administration. Previous study from Mann et al. (2010) and Pannu et al. (2007) show function improvements after administering statins orally. Testing oral simvastatin in a cervical unilateral contusion model may be able to demonstrate the beneficial effects.

Over the course of these experiments, I have realized the importance of a systematic approach. As it turned out, developing this injury model was not such a straight forward endeavor. In order to develop an injury model, it was not only important to control the three injury parameters, but it was also crucial to solve problems one at a time. The three challenges I was faced with were abnormal force spike, insufficient behavioural deficits and injury containment on the ipsilateral side of the spinal cord. These challenges were addressed one by one, as shown in Chapter 2 and it was this systematic approach that lead to the development of a novel cervical unilateral contusion model. In addition, to injure the animals consistently each time, I have simplified and standardized the steps to contusing each animal. The standardized procedure enables anyone to carry out the
injury which is inline with one of the original reasons of developing the IH impactor in the first place, (to be user-friendly and easily utilized by anyone).

Other cervical unilateral contusion model has already been introduced by Soblosky et al. (2001) using a modified Allen’s weight drop device and subsequently by Gensel et al. (2006) using the NYU impactor. Interestingly, these two injury models have not gained popularity even though there was a need for cervical SCI studies. This could be because when these two models were published, as mentioned earlier, SCI research was predominantly conducted on the thoracic level or because the popularity of other impactors such as the IH impactor started to grow. The severe settings for both models result in sufficient functional deficits, but the damage inflicted by the injury is not contained on the ipsilateral side in these two models. Recently, a unilateral cervical injury model using the IH impactor developed by Sandrow et al. (2008) has been reported. This injury model set the force at 200 kdyne to unilaterally injury the spinal cord; however, saline is poured into the surgical site to immerse the impactor tip. Although the force is set higher compared to the injury model presented in Chapter 2 of this thesis (150 kdyne), the actually force that is transferred to the spinal cord will most likely be significantly less as some of the energy will dissipate into the fluid before reaching the spinal cord.

To assess the overall preclinical evidence that is published, both positive and negative studies should be equally weighted and taken into consideration. A potential treatment must display an overall robust effect in animals if it is to have any chance of being efficacious in humans. Given the variables and variability of recovery in humans, it would be difficult for a treatment to display therapeutic benefits if its efficacy in rodents was dependent on precisely controlled conditions. Recently, a scoring system was published to assess the readiness of preclinical treatments. In this, a score is assigned to a given therapy based on the available published preclinical literature. The score is determined by the animal species that have been tested, injury models, level of injury, the demonstration of the “clinically meaningful” efficacy, and the reproducibility of the effect. Based on this scoring system, even after accounting the negative study presented here, minocycline scores the highest among the 14 promising therapies that are reported to have neuroprotective effects (Kwon et al., 2010b).

Currently there is an eminent need for an efficacious treatment for SCI, resulting in enormous research efforts in this regard. There are numerous preclinical therapies that are reported to be neuroprotective. With an urgent need for an effective treatment, these therapies face
challenges such as time, cost and limited patient pool in order to go through the process of clinical trials. The cost of clinical trial enrollment is currently $50,000 to $100,000 per person in the United States and Europe, and the projected cost of phase 2 preliminary efficacy trials in humans are no less than $5–10 million per candidate drug (Blesch and Tuszynski, 2009). This is complicated by the fact that human injuries are extremely variable. Unlike preclinical research, where experimental variables are tightly controlled, clinical trials leave these variables such as differences in age, gender, hormone state, concurrent illness, mechanism of SCI, severity of spinal cord compression, duration of spinal cord compression, type of surgical care, type of medical care, motivation, mood and differences between clinical trial sites, uncontrolled that introduce high variability that increase difficulty in detecting a treatment effect (Blesch and Tuszynski, 2009). Human SCI displays highly variable spontaneous recoveries as well. The current scale of the American Spinal Injury Association (ASIA) grading system to classify patients do not always accurately predict the outcome due to the variation of spontaneous recovery making enrolling patients for clinical trials difficult. It is reported that there are about 20% of ASIA A classified patients that demonstrate some sort of spontaneous recovery, 40% of the patients for ASIA B and 60% of patients for ASIA C by one year after injury (Blesch and Tuszynski, 2009). These factors require large numbers of patients in order to demonstrate a treatment effect and for a relatively low annual incidence of traumatic SCI, there are a limited pool of patients that can be recruited for testing (Fawcett et al., 2007; Geisler et al., 2001; Geisler et al., 2001; Lammertse et al., 2007). With these difficulties, we have to keep in mind that before entering clinical trial, we must carefully determine which therapies to translate.
4.4 References


APPENDIX
# Animal Care Certificate

**Application Number:** A05-1754  

**Investigator or Course Director:** Brian Kwon  

**Department:** ICORD  

**Animals:**  

| Rats Sprague-Dawley 669 |  

**Start Date:** January 2, 2005  

**Approval Date:** January 21, 2009  

**Funding Sources:**  

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<td>Neuroprotection for acute spinal cord injury: the preclinical evaluation of drugs that are currently used in human non-spinal applications</td>
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<tr>
<td>Wings for Life Spinal Cord Research Foundation</td>
<td>Pharmacologic neuroprotection in a cervical spinal cord contusion injury model</td>
</tr>
<tr>
<td>Wings for Life Spinal Cord Research Foundation</td>
<td>The preclinical evaluation of drug combinations as a neuroprotection strategy for acute spinal cord injury</td>
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<td>Funding Title:</td>
<td>Cerebrospinal Fluid Drainage and Cytokine Analysis in the treatment of Acute Spinal Cord Injury: A Pilot Study</td>
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<td>Cerebrospinal fluid drainage and cytokine analysis in the treatment of acute spinal cord injury: a pilot study</td>
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| Unfunded title: | N/A |

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

**A copy of this certificate must be displayed in your animal facility.**

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A07-0108

Investigator or Course Director: Brian Kwon

Department: Orthopaedics

Animals:

Rats Sprague-Dawley 83

Start Date: March 26, 2007
Approval Date: June 4, 2009

Funding Sources:

Funding Agency: Rick Hansen Man In Motion Foundation
Funding Title: Cerebrospinal Fluid Drainage and Cytokine Analysis in the treatment of Acute Spinal Cord Injury: A Pilot Study

Funding Agency: Rick Hansen Man In Motion Foundation
Funding Title: Cerebrospinal fluid drainage and cytokine analysis in the treatment of acute spinal cord injury: a pilot study
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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