PHARMACOLOGICAL NEUROPROTECTION FOR SPINAL CORD INJURY

by

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ABSTRACT

Spinal cord injuries can cause the catastrophic loss of motor and sensory function. The neurological deficits that result are the consequence of not only the primary injury to the spinal cord, but also a complex milieu of secondary pathological processes that are now beginning to be understood[1]. The major mechanisms that underlie this secondary pathology include vascular disruption, ischemia, oxidative stress, excitotoxicity, and inflammation[1]. In light of this, the fact that this secondary pathology occurs after the initial impact makes it potentially amenable to therapeutic intervention. Pharmacotherapies may attenuate some of these processes and minimize secondary damage.

Some of the promising treatments that are emerging for acute spinal cord injury are drugs that are already used by physicians for the treatment of unrelated diseases. These drugs, which have already been established to be safe for humans, offer the unique advantage over other novel therapeutic interventions that have yet to be tested in humans. This would save a tremendous amount of time and money needed for human safety studies, if considered as a treatment for spinal cord injury. Examples of such drugs include minocycline (an antibiotic), erythropoietin (a recombinant hormone used to treat anemia), and statins (a popular class of blood cholesterol reducers), all of which have demonstrated the ability to attenuate the various pathophysiological processes initiated after trauma to the central nervous system[2-10].

In a series of studies, erythropoietin, darbepoetin, atorvastatin, simvastatin, and minocycline were all evaluated for their ability to improve neurologic recovery in a clinically relevant model of spinal cord injury. My experiments revealed that erythropoietin, darbepoetin, atorvastatin and minocycline did not significantly improve neurological recovery. These negative results were in stark contrast to the positive findings which had been published in the literature suggesting that differences in experimental models and methodology influence the neuroprotective efficacy of these drugs. Simvastatin, on the other hand, demonstrated significant improvements in locomotor and histological outcomes. Although this is indeed exciting, the results were modest at best. My results highlight the need for further preclinical work on the above treatments to refine and optimize them prior to proposing them for human testing.

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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 Jae H.T. Lee, Anthea M.T. Stammers, and Hong-Moon Sohn for behavioral analyses. Histolgy, data analyses, and manuscript preparation was performed by myself, under the guidance of my graduate supervisor Dr Brian Kwon.

CHAPTER 1

Introduction[1]

[1] A version of this chapter has been published. Cody M Mann and Brian K Kwon, An Update on the Pathophysiology of Acute Spinal Cord Injury (2007), Semin Spine Surg 19:272-279.

1.1 INTRODUCTION

Trauma to the spinal cord often leaves patients with permanent and devastating losses. In an accident that occurs within milliseconds, the ability to feel, move, or even breathe can be taken, leaving an individual paralyzed for life. The debilitation that results places an immense burden on the unfortunate victims, who typically are young and healthy individuals in the prime of their lives[11]. The nature of this injury makes it one of the most devastating and tragic known to mankind.

Although much progress has been made in the immediate management and rehabilitative care of spinal cord injuries, there still is no effective therapy to treat paralysis. To date, only methylprednisolone has been approved for the treatment of spinal cord injury (SCI) and its efficacy has been severely questioned[1]. Clearly there is a need for better treatment options, and, accordingly, scientific and clinical research for SCI has continued to move on at an accelerated pace[1]. There is a vast repertoire of knowledge on the biology of spinal cord injury that has been acquired by basic scientists and clinicians over the years. This knowledge is extensive and has shown that pharmacological treatments can improve histological and neurological outcomes after SCI in animal models. It is our aim to develop a pharmacologic treatment for SCI that may ultimately be tested in human patients.

1.1.1 The Accident

The cause of a spinal cord injury (SCI) can range anywhere from community violence to an accident while engaging in recreational activity; however, the list of causes is topped by motor vehicle accidents[11,12]. It is estimated that there is an annual incidence of 15 to 40 cases per million throughout the world and 1200 new cases per year in the United States alone[11].

The injury begins when the vertebral column, which houses and protects the spinal cord, is subject to forces so great that it fails structurally. Thereafter, the malformed vertebral column and displaced bone/cartilage fragments are forcefully driven into the spinal cord, causing the so called “primary injury” to the cord.

Within seconds after the initial trauma, neuronal membranes are obliterated, blood vessels are damaged, and hemorrhage into the spinal cord parenchyma occurs[12]. The implications of these immediate events are significant. For instance, myelinated axons that travel to and from the brain conducting the electrical signals necessary for neurological function

are severed. And, as one would expect, the loss of axons appears to correlate well with the force of impact onto the cord[13]. The important thing to point out is that this primary injury does not typically severe the entire spinal cord, and that there are components of the cord at the injury site which are spared from the initial event. They become the target for further secondary damage, and the substrate for potential neuroprotective therapies.

1.1.2 Secondary Damage: The Injury Continues

Soon after the primary injury, a series of secondary events are triggered that go on to induce further damage to the injury site. A recent review by Norenberg et al described the histological changes that occur after human SCI and classified them according to how soon after the injury they occur[14]. The primary injury occurs at the time of impact, with the physical disruption of nervous and vascular tissue of the spinal cord. However, hours to days after SCI there are changes to neuronal and axonal structure, for example apoptotic death and axonal swelling respectively. Moving along the timeline, in the following days and weeks after SCI there is Wallerian degeneration, the anterograde degeneration of axons and myelin after axotomy, and similarly there is axonal dieback, the retrograde degeneration of axons and myelin[15,16]. In the end, anatomical abnormalities such as cavities and cysts present themselves within the spinal cord as a result of the accumulated cellular death and tissue loss[14].

1.2 THE PATHOPHYSIOLOGIC MECHANISMS OF SECONDARY DAMAGE

So far, the sequence of events that occurs after spinal cord injury has been described only from an anatomical and cellular perspective. Yet one can see that the primary insult to the spinal cord is only the beginning and the injury continues to evolve over time. At even closer glance however, it becomes evident that there is an explosion of cellular and molecular events that result in continued tissue destruction. The molecular and physiological mechanisms underlying secondary damage are a complex cascade of inter-related events. One of the largest contributors is ischemia, mediated by direct damage to the vascular networks that supply the spinal cord[17]. In parallel to vascular insult, the havoc that is imposed on neural tissue during the ensuing hours / weeks after injury is caused by excitotoxicity, oxidative stress, and inflammation[15,1]. The nature of each of these mechanisms and how they contribute to the injury is still incompletely understood.

The neurological impairment or paralysis one suffers from SCI is actually the result of the initial traumatic injury to the spinal cord plus the secondary damage that occurs over time. This is important because, in theory, if one could bring the "explosion" of secondary damage to a halt, neurologic function that would have been lost could potentially be preserved. This brings about the concept of "neuroprotection" or protecting nervous tissue from secondary damage. More patients are now surviving the initial SCI and a fewer number of patients are presenting complete injuries, likely a result of improved medical practice and patient care[11]. In most cases there is a residual amount of neural tissue across the lesion site that survives the secondary pathophysiologic and degenerative processes. This tissue, typically at the periphery of cord, consists of preserved white matter that surrounds a cavity[14,18]. Animal studies have showed that the number of surviving axons is highly correlated with neurological function, and that sparing even a relatively small amount of axons (ie. 5-10% of the total) can significantly increase recovery of locomotion after SCI[19-21]. Neuroprotection offers the potential of preserving neurologic function that – even if small – could be very meaningful to the quality of life for a SCI patient.

Since specific pathophysiologic mechanisms are thought to contribute to secondary damage, there is great interest in studying and better understanding them. They can serve as pharmacological targets that could potentially be inhibited to reduce secondary damage and improve neurologic function after SCI.

1.2.1 Vascular Abnormalities

Traumatic injury to the spinal cord usually leaves larger arteries lining the surface of the spinal cord, the anterior and posterior spinal arteries, intact. However, the intramedullary microvasculature is not so fortunate. Vascular damage typically occurs in the intramedullary centrifugal sulcal artery network that supplies most of the grey matter and white matter of the anterior, lateral, and posterior columns[22,23]. This is supported by the observation that hemorrhage in acute human spinal cord injury is most prominent in the grey matter, although it can be present anywhere[23,24].

Initial vascular abnormalities, as a result of direct mechanical damage, include hemorrhage, vasospasm, the release of vasoactive molecules, and the loss of pressure autoregulation. Subsequently, all these negatively influence circulation and perfusion, ultimately resulting in ischemia[24,25]. This starves the surrounding tissue from vital nutrients and comprises

survival. Extensive histological analysis of both human and animal spinal cords after injury indicates that much of the secondary cellular death that occurs is the result of damage to and malfunction of the vascular systems supplying the spinal cord[23,24]. For instance, necrotic tissue loss and cavitations that develop days to weeks after injury correlate strongly with hemorrhagic areas[24,26,17].

Secondary damage to the spinal cord that is caused by vascular abnormalities can be prevented to reduce neurological losses; this has been illustrated by the drug naloxone. Levels of endogenous opioid peptides, which serve as inhibitory neurotransmitters of pain, are increased after SCI[27,28]. Opioid peptides, such as Dynorphin-A, have negative effects on spinal cord blood flow through vasoconstrictory action, which can produce ischemia related neuronal death[27,28]. The treatment of experimental SCI with the non-specific opioid antagonist naloxone was observed to reduce the loss of blood flow that occurs after SCI and improve overall neurological recovery[29-32]. As a result, naloxone was tested as a treatment for acute human SCI in a clinical trial[33]. Some evidence was observed suggesting efficacy in incompletely injured patients and hence naloxone demonstrated that it could potentially improve the outcome of human SCI[33].

1.2.2 Excitotoxicity and Impairment of Ionic Homeostasis

All cells maintain specific extracellular and intracellular concentrations of such ions as calcium, sodium and potassium. This ionic homeostasis is required for normal cellular activity and survival. Furthermore, it is extensively used by the nervous system to propagate electric signals. Cells of the central nervous system (CNS) typically keep extracellular concentrations of sodium and calcium high and intracellular concentrations low. After a traumatic or ischemic injury however, intracellular sodium and calcium concentrations rise. Ionic homeostasis is largely controlled by the plasma membrane and its ability to regulate the movement of ions with membrane channels. Physical injury to the membrane or the malfunction of membrane channels can result in the loss of normal ionic homeostasis. For instance, after CNS injury the activity of the Na/K ATPase is reduced due to cellular energy losses (ie from ischemia) and so is the efflux of sodium from the cell[34]. Ligand gated channels also contribute ionic dysregulation[34]. Glutamate, which is the most abundant excitatory neurotransmitter in the nervous system, activates glutamate receptors such as the N-methyl-D-aspartate NMDA receptor and non NMDA α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/Kainate receptor. These are non-selective ion channels that transport calcium or sodium upon activation by glutamate.

Animal models indicate that, within minutes after SCI, glutamate is released and accumulates to abnormally high levels at the injury site[35]. Over-activation of the above receptors by excess glutamate goes on to contribute to the already declining ionic homeostasis and results in dangerously high levels of sodium and calcium within cells[36-39].

Ultimately, increased intracellular sodium and calcium levels are detrimental to cellular survival; several mechanisms can explain sodium toxicity. Increased intracellular sodium concentrations are almost certainly followed by water, resulting in cytotoxic edema[40]. High sodium concentrations within the cell can also cause the Na/H exchanger to come to an abrupt stop. This would lead to a decrease in intracellular pH and cytotoxicity caused by low pH would soon follow[41]. Lastly, there is the possibility that the Na-Ca exchanger may operate in reverse because of a reversed sodium gradient. This would increase intracellular calcium concentrations, secondary to increased cytoplasmic sodium[42]. Calcium can be detrimental to the cell though its own separate mechanisms, which will be discussed later. Several experimental studies have shown that by blocking the movement of sodium into cells with the sodium channel blocker tetrodotoxin (TTX), both histologic and neurologic outcomes can be improved after SCI[41,43-45]. Rosenberg and Wrathall demonstrated that sodium toxicity occurs immediately after SCI, as TTX looses its neuroprotective effects when applied 4 hours after SCI[46]. A more clinically practical sodium channel blocker, riluzole, can be delivered safely through a systemic route. Studies on rats have shown that riluzole can indeed reduce secondary white and grey matter damage and increase neurologic recovery[47]. The Food and Drug Administration has now approved riluzole for the treatment of ALS, and a clinical trial to test its efficacy for human SCI is in its late stages of planning.

As mentioned, an increased intracellular calcium concentration also plays a very important role in cellular injury. High levels of intracellular calcium disrupt the many cellular processes that are controlled by calcium. Calcium regulated proteases and endonucleases, for instance, can be over activated to initiate protein and DNA degradation[48]. Calpains are a family of calcium dependent proteases that are involved the degradation of cytoskeletal protiens; when overactive they destabilize cellular structure and lead to neuronal death[49]. Calpain specific inhibitors can reduce secondary damage after SCI in animal models[49]. In addition to overactive enzymes, mitochandria can absorb calcium when intracellular concentrations are high. Eventual mitochondrial overload of calcium leads to mitochondrial swelling and finally an assembly of pores in the mitochondrial membrane. This, in turn, releases mitochondrial contents into the

cytoplasm, halts ATP production, and increases in the production of radical oxygen species; all of which contribute to cell death via necrosis or apoptosis (discussed later)[48].

As stated earlier, much of the sodium and calcium dysregulation that occurs is through the over activation of glutamate receptors. Recognition of the important role of glutamate excitotoxicity after CNS injury has prompted much interest in the pharmacological blockade of NMDA and non-NMDA receptors as a means of protecting the susceptible spinal cord tissue. NMDA and AMPA glutamate receptors can be blocked by antagonists gacyclidine (GK11) and NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) respectively. With these pharmacological interventions the excessive influx of calcium and sodium and ensuing toxicity can be attenuated. For example, in animal models of SCI, AMPA receptor antagonists have been shown to reduce white matter loss and decrease functional deficits[35,38]. NMDA receptor blockade has also been extensively studied in animal models[37,38,50-54] and enthusiasm over promising laboratory results initiated a prospective randomized human clinical trial of an NMDA receptor antagonist gacyclidine (Ipsen-Beaufour, Les Ulis, France)[55]. Unfortunately, the results of this study (unpublished at this time) did not reveal any neurologic benefit for human SCI.

1.2.3 Oxidative Stress

Molecules that contain one or more unpaired electrons are referred to as free radicals and include reactive oxygen species such as the superoxide radical (O_2-), the hydroxide radical (OH-), and peroxynitrite (ONOO-). These are often created by inefficient electron transport during mitochondrial energy metabolism. For instance, superoxide (O_2-) is made by the addition of a single electron to oxygen. Superoxide can then become the extremely reactive hydroxide radical (OH-) by the addition three more electrons[56]. Because the production of free radicals is so intertwined with energy metabolism, vascular related ischemia and subsequent reperfusion induces the production of an enormous amount of free radicals after CNS injury[57]. Cellular enzymes can also be source of reactive oxygen species. Xanthine oxidase, nitric oxide synthase (NOS), and phospholipases can all produce free radicals. For example, nitric oxide (NO) produced by NOS can react with O_2- to from the free radical ONOO- (peroxynitrite), which is also a potent oxidant[56]. Interestingly, each of these enzymes can be activated by increases in intracellular calcium[58-60]. Therefore ionic disturbances (intracellular calcium) initiated by CNS injury can indirectly activate enzymes such as NOS and increase the production of free radicals.

This highlights how complex the production of free radicals is; it is very much interrelated with ischemia, excitotoxcity, and inflammation (discussed later).

The production of reactive oxygen species occurs in a domino like effect, being augmented by the various secondary injury mechanisms. Free radicals compromise cellular survival through the oxidation of essential cellular elements. For example, polyunsaturated fatty acid side chains in cellular membranes are damaged in chain reaction by lipid peroxidation. This begins when an oxidant such as hydroxide (OH-) or peroxynitrite (ONOO-) reacts with and extracts a hydrogen atom from a fatty acid chain[61]. This, in turn produces more radicals that react with more fatty acid side chains. This self-propagating reaction reoccurs again and again to slowly dismantle the cellular membrane, contributing greatly to membrane malfunction and cell death[56,61]. Free radicals can also act on free or protein bound amino acids. Oxidation has the potential to deform structural, receptor, and enzymatic proteins[56]. Lastly, DNA is also susceptible to oxidation. Oxidative damage to DNA can result in DNA-DNA cross-links, DNA-protein interactions, and DNA strand breaks, all of which can damage DNA and lead to cellular death[56].

Given the relevance of oxidative stress in secondary damage and a plethora of available compounds that act as antioxidants, there has been significant interest in applying such antioxidants as neuroprotectants in acute spinal cord injury. The attenuation of lipid peroxidation was, for example, one of the primary biological rationales behind the use of high dose methylprednisolone as a neuroprotective agent after spinal cord injury. Products of lipid peroxidation increase after spinal cord ischemia and increase even further during vascular reperfusion[62,63]. Innumerous compounds have shown promise at reducing lipid peroxidation and improving neurologic recovery in animal models of spinal cord injury[64-67]. Some, such as melatonin[68] and polyethylene glycol[69], are already well tolerated in humans and as such may be candidates for clinical evaluation in the near future.

1.2.4 Inflammatory / Immunologic Response

An injury to the spinal cord is followed by an acute inflammatory response that is thought to be an important component to secondary damage. While it is evident that ischemia, excitotoxicity, and oxidative damage are interrelated, the central role of inflammation in secondary damage after spinal cord injury is highlighted in a recent review, in which Jones and colleagues state that *"most (if not all) of these secondary degenerative processes can be initiated*

and/or coordinated by cellular and humoral components of posttraumatic inflammation"[70]. Given that inflammation is an important aspect of secondary damage after spinal cord injury, and that technology currently exists to influence it (as evidenced by the wide assortment of anti-inflammatory drugs available at the local pharmacy), it is only logical that a significant amount of scientific interest would be directed at developing therapies to inhibit posttraumatic inflammation after spinal cord injury, with the hopes of minimizing its deleterious effects.

The cellular aspects of the inflammatory and immune response after spinal cord injury include microglia (resident CNS macrophages), circulating peripheral macrophages, neutrophils, and lymphocytes. The precise role of immune cells in secondary damage after SCI is still unknown and how much these cells contribute to the injury remains controversial. Neutrophils are the first cells to migrate into the injured spinal cord after injury. They are thought to contribute to cellular damage through the production of reactive oxygen and nitrogen species (contributing to oxidative stress) and matrix metalloproteinases (contributing to degradation of adjacent extracellular matrix). The activation of microglia and the invasion of bloodborne macrophages and T-lymphocytes occur within a week post injury and continue to the chronic stages of SCI at a lower capacity[71,72]. It is clear that macrophages and activated microglia contribute to local phagocytosis after SCI[72,73]. There is a spatial and temporal correlation between populations of these cells within the injured spinal cord and posttraumatic demyelination, axonal degeneration, and cavitation, which is suggestive albeit not conclusively indicative of a causative role[72,74,75]. An injurious role to macrophages and neutrophils is indicated by the fact that blocking their activation and migration into the spinal cord appears to be neuroprotective in animal models[74,76-78].

While these observations point to a deleterious effect, there is also evidence to suggest that macrophage activation may be part of a reparative and potentially regenerative response. Following axotomy in the peripheral nervous system (PNS) there is a more robust inflammatory response as compared to the CNS. In the PNS, macrophages play an important role in clearing debris through phagocytosis and promoting a permissive growth environment for subsequent regeneration through the production of growth factors. This is thought to contribute to the superior ability of the PNS to regenerate[79-82]. Work from Michal Schwartz in Israel and others has suggested that macrophages activated by exposure to peripheral myelin and transplanted into the spinal cord after injury may in fact promote repair and regeneration[81,83,84]. This prompted the initiation of a human clinical trial in which autologous macrophages were harvested, stimulated ex vivo, and then implanted into the injured spinal cord. The results of this Phase I study were

published in 2005 and indicated that some motor, sensory, and electrophysiologic function did improve in this small sample of eight patients[85]. A Phase II study of this treatment was launched, however was suspended due to the enormous financial costs of conducting this study[86].

The role that T-lymphocytes play in the overall outcome of spinal cord injury remains even more elusive. Major histocompatability (MHC) class II molecule expression has been detected in the spinal cord after injury[72]. In the periphery, MHC molecules serve as antigen presenting proteins that allow T-lymphocytes to mount an "adaptive" immune attack against foreign antigens. The presence of T-lymphocytes and MCH class II proteins in the CNS, after injury, does not necessarily signify the induction of adaptive immunity or an autoimmune reaction[86]. T-lymphocytes clearly have the potential to be destructive in the injured spinal cord[86]. Yet, T-Lymphocytes, just as macrophages, have demonstrated both beneficial and reparative characteristics[86]. Vaccines that initiate an accelerated T-Lymphocyte response against self-antigens have given rise to the idea of protective autoimmunity. A mild autoimmune response in the CNS can apparently be used to take advantage of T-lymphocytes and their ability to regulate inflammation and produce reparative neurotrophic factors[86].

The cellular components of inflammation are regulated by a barrage of cytokines, chemokines, growth factors, and other soluble molecules. Inflammatory cells, astrocytes, microglial cells, and endothelial cells all communicate with each other by producing these signaling molecules[87-90]. Several cytokines have been demonstrated to have important roles in mediating secondary injury[91]. In one study, IL-1, IL-6, and TNF-α were determined to be pro inflammatory, increasing the number of macrophages and activated microglial cells at the injury site[91]. On the other hand, IL-10 has been defined as an anti-inflammatory and neuroprotective cytokine. Administering IL-10 reduced overall tissue damage and improved functional recovery after experimental SCI[92]. Despite these important experiments, it often seems as if cytokines are too quickly labeled as "good" or "bad". Similar to the cellular constituents of inflammation, the role of certain cytokines as pro- or anti-inflammatory agents is often cloudy, and whether a certain cytokine is deemed to be deleterious or beneficial is often related to its temporal and spatial expression, and perhaps most importantly, how it is tested.

TNF-α, for example, is believed to have both protective and destructive effects in the CNS. It is expressed within minutes after spinal cord injury, particularly in neurons, glial cells, and endothelial cells[92-95]. TNF-alpha/TNF-alpha receptor signaling results in the activation of transcription factor NF-kB. This consequentially increases the expression of such genes as iNOS, other pro-inflammatory cytokines, and adhesive molecules that facilitate the infiltration of

leukocytes[96]. A recent study reported that the TNF-α antagonist etanercept (marketed as Enbrel for the treatment of rheumatoid arthritis) decreased inflammation, apoptosis, and functional deficit after spinal cord injury[97]. However, in contrast, TNF-α has also been reported to stimulate antioxidant pathways[98] attenuate increases in intracellular calcium concentration, and reduce cell death[99-101]. Hence, this particular cytokine is believed to have both protective and damaging effects, mirroring the situation with macrophages and T-Lymphocytes as described above.

Such research has thus revealed that the inflammatory process is extremely complex. It is now apparent that to view posttraumatic inflammation as merely a unidimensional response in need of inhibition is a gross oversimplification. There are components of the inflammatory and immune response that promote secondary injury, while there are other components (or the same components at work at different times postinjury) that promote repair and regeneration[102].

1.2.5 Cellular Death

Ultimately, all the aforementioned pathophysiologic processes of vascular disruption and ischemia, excitotoxicity, oxidative stress, and inflammation contribute to the demise of neuronal and glial cells at the injury site. This cellular death comes in two forms: necrotic cell death, and apoptotic, or programmed, cell death. Both are seen after spinal cord injury. Necrotic cell death occurs after a severe injury overwhelms the cell's homeostatic mechanisms, leading to membrane and organelle damage, the loss of ATP production, and the passive swelling and eventual disruption of the cell. In contrast to this, apoptotic cell death is an active process that the cell itself participates in. Apoptosis is mediated by the activation of protease enzymes called caspases, which target cytoskeletal and nuclear proteins. Because necrosis is a passive process by which the cell succumbs to the injury, it is metaphorically akin to a "cellular homicide." Apoptosis, or "programmed cell death," is an active, energy-dependent process that makes it akin to a "cellular suicide." Morphologically, the manner in which this death occurs is distinct, with necrotic cells swelling, lysing, and releasing their intracellular constituents, thus inciting a local inflammatory reaction. Alternatively, apoptotic cells shrink, fragment, and are cleared by phagocytosis without a significant inflammatory reaction[103].

The practical reason for distinguishing between necrotic and apoptotic death is related to the potential for intervening in the latter. Necrotic cell death at the injury site is thought to occur quite early, likely as the result of severe tissue disruption at the epicenter of injury—hence, there may be little that we can do to stop this from occurring. Apoptosis, however, can occur in the

ensuing days to weeks, and because it is an active process mediated by specific enzymatic pathways, it can be potentially influenced by treatments. Apoptotic cell death of oligodendrocytes has been demonstrated in rats, monkeys, and humans after spinal cord injury[104-107]. Therefore, targeting apoptosis may be a rational strategy in preserving myelin and facilitating action potential conduction across the injury site. For instance, interventions that block the activation of caspases or their downstream targets may be promising for reducing apoptotic cell death after spinal cord injury. Several caspase inhibitors have been reported to prevent apoptosis in vitro. Their use in experimental SCI however has presented conflicting results[49,106,108,109]. Nevertheless, the development of caspase inhibitors into clinically relevant therapies for spinal cord injury is still in the experimental stages[49,106,108,109] and further research is promising.

1.3 EMERGING NEUROPROTECTIVE THERAPIES

Observations of the natural recovery that occurs after SCI in humans and animals have fueled much of the excitement in SCI research. It has been noted that even with a small number of axons spanning the lesion site there is significant motor recovery[110]. In monkeys, preserving less than 25% of white matter in the thoracic spinal cord allows injured subjects to recover use of lower limbs[110]. In humans, even as little as 10% can leave a patient with residual motor abilities[110]. Together this suggests that sparing or regenerating even a small number of tracts across the lesion site can make a world of difference in the life of a paralyzed individual. Several studies up to date have experimented with the concept of regeneration within the spinal cord[111]. In short, the use of growth promoting factors, cells, or chemical agents are able to "regenerate" certain tracts depending on treatment type[111,112]. However, the functional significance of axonal regeneration in these studies remains unknown and numerous problems still lie ahead in trying to regenerate within CNS[112].

In light of the challenges associated with the promotion of de novo axonal regeneration within the CNS, it may be viewed that the strategy of "neuroprotection" to spare neuronal matter from secondary damage is more imminently feasible. As illustrated earlier, several different pathophysiologic targets exist; each of these pathologic mechanisms can be attenuated to increase neurologic recovery. Clearly, tremendous efforts are being made to develop therapies that might be neuroprotective in acute spinal cord injury. From a clinical perspective, the need for such therapies is quite urgent. Although some degree of spontaneous recovery is seen in

many SCI patients, most have permanent deficits and, sadly, there are currently no treatments available that have been shown to reverse or minimize this.

One of the promising areas of research in the development of such therapies is in the investigation of drugs that have neuroprotective effects but are also already in human clinical use for typically unrelated indications. Such drugs have the appeal that their safety and tolerability in humans is already well established, and thus, they will not be mired in years of safety testing that would be necessary for a treatment that, for example, involved viral gene transfer or stem cell transplantation.

1.3.1 Erythropoietin

Erythropoietin (Epogen; Amgen), a glycoprotein hormone, is known for its ability to regulate the proliferation and differentiation of erythroid precursor cells (erythropoiesis). Recently, much evidence has supported the additional role of erythropoietin as a neuroprotectant[113]. The protective action of erythropoietin seems to involve a number of cellular pathways influenced by the erythropoietin receptor, which is also expressed in the central nervous system[113]. In short, erythropoietin is able to reduce oxidative damage, excitotoxicity, the expression of pro-inflammatory cytokines, and apoptotic cell death (reviewed by Bartesaghi and coworkers)[113]. This has been demonstrated in several animal models of spinal cord injury (Table 1.5.1), thus supporting the neuroprotective role of erythropoietin from a functional and histological standpoint[114-118]. Human clinical trials evaluating erythropoietin in acute treatment of spinal cord injury have not yet been initiated. However, a recent clinical trial investigating erythropoietin as a treatment for stroke was promising and demonstrated that erythropoietin can be safely used in humans[119].

1.3.2 HMG-CoA Reductase Inhibitors "Statins"

The statins are a group of drugs that inhibit hydroxymethylglutaryl- coenzyme A (HMG-CoA) reductase, the enzyme responsible for the conversion of HMG-CoA to mevalonate, the rate-limiting step in de novo cholesterol synthesis[120]. They are the most commonly prescribed class of lipid-lowering drugs today and include lovastatin (Mevacor; Merck & Co, Inc, Whitehouse Station, NJ), atorvastatin (Lipitor; Pfizer Inc), pravastatin (Pravachol; Bristol-Myers

Squibb, New York, NY), and simvastatin (Zocor; Merk & Co, Inc). It has been recently recognized that the statins also have powerful immunomodulatory and anti-inflammatory effects[121]. Statins have been shown to inhibit the expression of a variety of pro-inflammatory cytokines such as monocyte chemotactic protein (MCP)-1, IL-2, IL-12, and interferongamma[122,123]. Pannu and coworkers reported that the administration of atorvastatin 1 week before spinal cord injury resulted in a significant reduction of inflammatory cytokines, neuronal cell death at the injury site, and a significant improvement in locomotor scores[124]. The efficacy of statins has been demonstrated when administered after spinal cord injury, and also in several other experimental models of neurological injury (Table 1.5.3). Further preclinical work is needed on this class of lipid-lowering agents, but their widespread use certainly makes them appealing candidates for human evaluation in spinal cord injury.

1.3.3 Minocycline

Minocycline (Minocin; Wyeth, Madison, NJ) is a tetracycline antibiotic that has neuroprotective properties independent of its ability to inhibit protein synthesis (its mode of antimicrobial action). Minocycline has been found to attenuate the inflammatory events after CNS trauma. Evidence exists to suggest that minocycline inhibits microglial activation, attenuates the release/activity of pro-inflammatory mediators (cytokines, reactive oxygen species, and matrix metalloproteinases), and limits cell death pathways[125]. This neuroprotection has been demonstrated by independent laboratories in various animal models of spinal cord injury (Table 1.5.4), in which minocycline administration was associated with reductions in lesion size, diminished oligodendrocyte apoptotic death, and improved functional outcome[10,126]. A human clinical study evaluating minocycline for the acute treatment of spinal cord injury has now begun in Calgary Alberta, Canada, and results of this prospective randomized controlled trial are eagerly awaited.

1.4 OBJECTIVES

A quick Pub Med search with the term “neuroprotection and spinal cord injury” will reveal hundreds of studies and hundreds of different treatments that could potentially be used to treat spinal cord injury. Many of these have been tested on humans, only to reveal they are unable to reproduce the results seen in experimental studies. As a result, only methylprednisolone has been

approved for the treatment of spinal cord injury, and even it has had its efficacy questioned; many clinicians have now abandoned its use. I have chosen several neuroprotective agents that have demonstrated the potential to improve neurological outcomes and are "safe to use" in humans. My objectives are to evaluate or reproduce their therapeutic efficacy in a clinically relevant model of spinal cord injury and compare them with each other to find an optimal treatment, one that would have the greatest likelihood for success in a clinical trial.

1. Determine if erythropoietin can attenuate secondary damage and increase neurological recovery in a clinically relevant model of spinal cord injury, and compare these results with darbepoetin, a derivative of erythropoietin.

2. Compare two pharmacologically different statins, simvastatin and atorvastatin, in their ability to attenuate secondary damage and increase neurological recovery in a clinically relevant experimental model spinal cord injury.

3a). Enhance the neuroprotective effect of simvastatin in experimental spinal cord injury by extending its dosing regimen.
3b). Determine if the beneficial effect of simvastatin on experimental spinal cord injury can be augmented by concurrent minocycline administration.

Each objective, 1, 2, and 3 will be addressed in chapters 2, 3, and 4 respectively. 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. The experimental model of spinal cord injury that we have chosen to use is the, well-validated, thoracic contusion of the rat spinal cord. This produces a progressive injury that develops over time with the pathological mechanisms described above[127]. Furthermore, the spinal cord is incompletely injured and rats exhibit significant recovery in the use of their hind limbs, which can be subsequently evaluated using a number of behavioral tests, which have also been validated[127]. Overall, the contusive injury model is thought to best stimulate the biomechanics and pathology of human spinal cord injury[127]. These injuries will be performed using an Ohio State University (OSU) Impactor, which is supposed to consistently deliver a blunt force to the spinal cord using a computer feedback-controlled electromechanical device[127].

1.5 TABLES

Table 1.5.1 The evaluation of erythropoietin for spinal cord injury (Page 1/5)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Gorio et al. PNAS, 2002[5].	Female SD rats 240-260g	T9 contusion with a modified Univ of Trieste Impactor	Recombinant human erythropoietin, (rhEPO) via IP injection. 500 units/kg/day or 5000units/kg/day	1h after injury, up to 7 days post injury	1. Single 5000units/kg dose 1h post injury (n=14) 2. Daily 5000units/kg dose starting 1h post injury, for 7 days (n=14) 3. Daily 500units/kg dose starting 1h post injury, for 7 days (n=14) 4. Saline injection 1h post injury (n=14) Half of the animals in each group sacrificed for anatomical study at 7 days.	Significant improvements in locomotor recovery observed. A single 5000unit/kg dose was just as effective as daily dose for 7 days and both these were more effective than a daily 500unit/kg injection. BBB scores, at day 28, were roughly 18 for the EPO treated (5000units/kg) and 10 for saline (n=6/group). Swimming scores were also significantly higher at 7 days post injury for the EPO group (n=12/group).	7 days after injury, EPO treatment (daily for one week) resulted in a significantly smaller cavitation volume (~25%) at and surrounding the epicenter. (n=12/group). Rostral to the epicenter, the number of apoptotic cells (TUNEL) was also significantly reduced by daily EPO treatment. (n=12/group).
Brines et al. PNAS, 2004[128].	C57/BL 6 WT and βcR KO mice (8-16 weeks old)	T3 Spinal cord compression with steel rod for 4mins.	Single dose of EPO or carbamylated erythropoietin (cEPO) (10μl/kg) given by IP injection	Single dose immediately after injury.	1. cEPO WT (n=10) 2. EPO WT (n=10) 3. PBS WT (n=10) 4. cEPO KO (n=10) 5. EPO KO (n=10) 6. PBS KO (n=10)	The BMS locomotor scale was used to evaluate locomotion. EPO or cEPO mediated complete locomotor recovery in WT mice, however this was not seen in the βcR KO mice or PBS controls.	Several other independent experiments were used to confirm that the βc-Receptor (βcR) mediates EPOs protective effect.
Kaptanoglu et al. Neurosurg Rev., 2004[6].	Male Wistar Rats 215 – 260g	T7-T8 weight drop contusion. 50g - cm	Single dose of EPO (5000 IU/kg, 1000 IU/kg, 100 IU/kg) given by IP injection.	Single dose immediately after injury.	1. Vehicle Control (n=8) 2. 100 IU/kg (n=8) 3. 1000 IU/kg (n=8) 4. 5000 IU/kg (n=8) 5. MPSS 30mg/kg (n=8) Animals sacrificed 2h post injury –	None	2h post injury – thiobarbituric acid-reactive substances were used to estimate lipid peroxidation. Out of all the treatments, EPO (5000 IU/kg) reduced peroxidation and protected the spinal cord the greatest.

Table 1.5.1 The evaluation of erythropoietin for spinal cord injury (Page 2/5)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Gorio et al. PNAS, 2005[129].	SD rats. Gender Not specified 240 – 260g	T9 contusion with a modified Univ of Trieste Impactor	Single dose of rhEPO (5000, 500, 100 units/kg) given by IP or IV injection.	Single dose given at various times after injury: 30min, 24h, or 48h after injury.	Each group had at least 18 animals 1. EPO 5000 units/kg i.p. (30min) 2. EPO 500 units/kg i.p. (30min) 3. EPO 500 units/kg i.v. (30min) 4. EPO 100 units/kg i.v. (30min) 5. EPO 5000 units/kg i.p. (24h) 6. EPO 5000 units/kg i.p.(48h) 7. EPO 5000 units/kg i.p. + MPSS 30 mg/kg, i.p. (30min) 9. MPSS (30 mg/kg, i.p.) 10. Saline	BBB locomotor scores revealed that optimal effects of EPO treatment were achieved with 5000 units/kg i.p. or 500 units/kg i.v. given 30min after injury. Scores at 28 days for these two groups were: 13 for EPO treated and 9 for Saline treated. Delay in treatment up to 48h after injury yielded a small benefit that lasted only for the first 3 weeks. Co treatment with MPSS negated the positive effects of EPO treatment.	Histologic analysis of the spinal cord suggests that EPO at 5000 units/kg i.p. is able to significantly increase the amount of spared tissue at and around the injury epicenter. This neuroprotection was negated with MPSS co treatment. Both MPSS and EPO equally reduced pro-inflammatory cytokines MIP-2, TNF-α, Il-1β, and IL-6.
Boran et al. Restor. Neurol. and Neurosci., 2005[130].	Male Wistar rats 180-220g	T6-T7 weight drop injury 50g-cm	Single dose of rhEPO, 5000 IU/kg, given by IP injection.	Single dose of EPO was delivered 1h post injury.	1. MPSS (30mg/kg i.p 60min post injury) (n=20) 2. rhEPO (n=20) 3. No treatment. (n=20)	A swimming test was used to evaluate motor recovery after injury. 14 days post injury, only EPO increased motor recovery; significant improvements began at day 4 and were observed until the experimental endpoint of day 14.	None.
Cetin et al. Eur Spine J 2006[131].	SD rats 200 - 300g	T3 compressive injury using an aneurysm clip (0.6N force for 1min)	A single dose or 3 multiple doses of rhEPO, 1000 IU/kg given by IP injection.	Doses started 5min or 40min after injury	N=8 per group 1. Control 2. EPO, Single dose 5min post injury 3. EPO, Three daily doses starting 5 min post injury 4. MPSS (30mg/kg i.p. at 5min post injury + 8h i.p infusion at 5.4mg/kg) 5. EPO, single dose 40min post injury + MPSS 6. EPO, Three daily doses starting 40 min post injury + MPSS	Locomotor recovery was analyzed using the swimming test 24h and 72h after injury. A daily dose of 1000 IU/kg for 3 days promoted better behavioral recovery than a single 1000 IU/kg treatment. Contrary to Gorio et al. (2005), the combination of three 1000 IU/kg doses with Methylprednisolone provided the best results.	Histological scores indicated that a daily dose of 1000 IU/kg for 3 days preserved tissue better than a single 1000 IU/kg treatment. However, a combination of three 1000 IU/kg doses with Methylprednisolone provided the best results

Table 1.5.1 The evaluation of erythropoietin for spinal cord injury (Page 3/5)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Grasso et al. J Neurosurg Spine 2006[132].	SD rats 275 – 300g	T3 compressive injury (Clip with 58g force for 1min)	asialoEPO (10μg/kg) EPO (10μg/kg) given by IV injection.	Doses started 24h before injury or immediately after injury.	1. asialoEPO 24 before injury (n=6) 2. asialoEPO immediately after injury (n= 6) 3. 3 asialoEPO daily doses and then 2/week (n=6) 4. EPO 24 before injury (n=6) 5. EPO immediately after injury (n= 6) 6. 3 EPO daily doses and then 2/week (n=6)	BBB analysis for 6 weeks post injury revealed that EPO treatment at the time of injury (~16) was superior than EPO pretreatment (~13) at improving locomotor recovery when compared to saline control (~9). No benefit was observed from administering multiple doses of EPO as opposed to just one at the time of injury	EPO administration increased GFAP expression (western blot) and the number of GFAP positive cells (immunoreactivity). Serum analysis confirmed that EPO must be present within systemic circulation at the time of injury.
Arishima et al. Spine 2006[133].	Male Wistar rats 380 - 500g	T8-T9 Compressive injury (120g weight lowered onto cord for 2 mins)	EPO (5000 IU/kg) given by IP injection.	Doses given 15min and 24h after injury.	Animals sacrificed at 0, 6,12 hours and 1,3,5,7, days after injury. Each end point contained the following groups: 1. EPO given 15min and 24h post injury (n=4) 2. Saline control (n=4)	None.	The number of apoptotic cells (believed to be mainly oligodendrocytes and motor neurons) was significantly reduced with EPO treatment (assessed by TUNEL or activated caspase3 positive cells). These anti apoptotic effects were observed up to 7days post injury. Cavitation volume was also reduced at the injury epicenter (H&E stain).
Vitellaro - Zuccarello et al. Neuroscience 2007[134].	Male SD rats 240 – 270g	T9 contusion with a modified Univ of Trieste Impactor	EPO (5000 IU/kg) given by IP injection.	Single dose given 30min after injury	1. Saline control (n=7) 2. rhEPO (n=7)	28 days after SCI, the BBB locomotor scores were ~8 and ~15 for the control and EPO groups respectively. This difference was statistically significant.	Spinal cords were analyzed at 4weeks post injury. EPO preserved ventral white matter compared to saline controls. The number of NG2 expressing oligodendrocyte precursor cells in the cord was increased in response to EPO treatment. Lastly, phosphacan immunoreactivity was reduced and 5HT immunoreactivity was increased with EPO treatment

Table 1.5.1 The evaluation of erythropoietin for spinal cord injury (Page 4/5)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Vitellaro - Zuccarello et al. Neuroscience, 2008[135].	Male SD rats 240 – 270g	T9 contusion with a modified Univ of Trieste Impactor	EPO (5000 IU/kg) given by IP injection.	Single dose given 30min after injury	1. Saline control (n=14) 2. rhEPO (n=14)	28 days after SCI, the BBB locomotor scores were ~8 and ~15 for the control and EPO groups respectively. This difference was statistically significant.	EPO treatment was associated with an increase in AQP4 immunoreactivity and a decrease in GFAP immunoreactivity. These authors also reported that EPO causes an increase in the total volume occupied by micro-vessels in the injured spinal cord.
Okutan et al. J. Clin. Neurosci., 2007[136].	Female Wistar rats 210 – 250g	T8 weight drop contusion (40g-cm)	rhEPO (1000 IU/kg) given by IP injection.	Single dose given immediately after injury.	1. Vehicle Control (n=8) 2. MPSS (30mg/kg) i.p., single dose (n=8) 3. EPO (1000 IU/kg) i.p (n=8) Animals killed 24h after injury	BBB scores, 24 hours after injury, were reported. EPO treatment increased locomotor recovery (~8) when compared to MPSS (~4) or vehicle control (~3).	24 hours post injury MPO activity (neutrophil infiltration) was equally decreased by MPSS or EPO treatment. Activated caspase3 activity (apoptosis) was also reduced by similar amounts by either drug.
King et al. Eur. J. Neurosci. 2007[137].	Male Wistar rats 200 – 220g	T10/T11 Unilateral Hemi-section	cEPO or rhEPO (40μg/kg) given by IP injection.	Two injections given 30min and 24h after injury.	1. Vehicle control (n=6) 2. EPO (n=6) 3. cEPO (n=6) Animals killed 3days after injury.	None.	Lesion size was significantly reduced by both cEPO and EPO. In addition, TUNEL (apoptosis) and β-APP (damaged axons) staining was decreased around the lesion site in response to either EPO or cEPO treatment. Schwann cell infiltration was increased with either treatment, however macrophage infiltration remained unchanged.
Mann et al. J. Exp. Neurol, 2008[138].	Male SD rats 320 – 340g	T9/T10 Contustion with the Ohio State University (OSU) Impactor (200-260 kdyn)	rhEPO (5000 IU/kg) or Darbepoetin (10mg/kg) given by IV injection.	Single injection given 1h post injury.	1. Vehicle control (n=11) 2. EPO (n=12) 3. Darbepoetin (n=11)	The BBB, hindpaw sensory, footfall, and footprint analyses were performed. Treatment with either EPO or Darbepoetin did not yield any significant improvements in neurological recovery.	Histologic analysis (EC stain) of white and grey matter did not show any significant neuroprotective effects associated with either EPO or Darbepoetin.

Table 1.5.1 The evaluation of erythropoietin for spinal cord injury (Page 5/5)							
Authors	**Animal Model**	**Injury Model**	**Intervention**	**Intervention Timing**	**Experimental Groups**	**Behavioral Outcome**	**Histologic / Biochemical/ Physiologic Outcome**
Pinzon et al. .J. Exp. Neurol, 2008[139] .	Female Wistar rats (220-280g) (clip compression model) Or Female Sprague-Dawley (240-260g) (Contusion model)	T3 clip compression (20g for 10s) T9, MASCIS (NYU) weight drop contusion (10g from a height of 12.5mm)	1000 IU/kg rhEPO given by IP injection.	Single dose and multiple doses immediately after injury.	Clip Compression 1. 1000 IU/kg rhEPO IP immediately after injury (n=15) 2. 1000 IU/kg rhEPO IP immediately after injury, followed by a dose at 24h and 48h (n=15) 3. Vehicle (saline) (n=15) Weight drop contusion 1. Single dose of 5000 IU/kg rhEPO. 2. 5000 IU/kg rHEPO IP immediately after injury and every 24 hours for 7 days. 3. Saline	rhEPO did not improve the locomotor behavior of injured rats in either injury model, weight drop contusion or clip compression.	rhEPO did not significantly improve secondary changes after either form of spinal cord injury (weight drop contusion or clip compression). Necrotic changes and cavitation were similar in both treated and control animals.

Table 1.5.2. The evaluation of darbepoetin for different forms of neurological injury

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Belayev et al., Stroke, 2005[140].	Adult male Sprague-Dawley rats (277-339g)	**Cerebral ischemia:** 2-hour middle cerebral artery (MCA) occlusion	Darbepoetin (10μg/kg) delivered intraperitoneally (IP).	Darbepoetin delivered at the time of reperfusion (2-hours after MCA occlusion)	3 day survival 1. Vehicle, n=7. 2. Darbepoetin n=8. 14 day survival 1. Vehicle, n=6. 2. Darbepoetin, n=8.	Darbepoetin treated animals had improved neurological scores starting 1 hour after treatment and lasted until the 14-day endpoint.	Darbepoetin decreased ischemic brain damage; infarct areas were significantly decreased with treatment.
Villa et al., J Cereb Blood Flow Metab., 2007[141].	Male Crl:CD(SD)BR rats (250-285g)	**Cerebral ischemia:** Blood flow through the middle cerebral artery (MCA) was blocked for one hour.	Carbamylated Darbepoetin (50μg/kg) delivered intravenously (IV) Also looked at carbamylated erythropoietin (CEPO) and erythropoietin.	Carbamylated darbepoetin was delivered at various times after MCA occlusion.	24 hour survival, treatment one hour after MCA occlusion 1. Darbepoetin, n=9 2. CEPO, n=8 3. Vehicle, n=9	Sensimotor scores (De Ryck limb placement test) were significantly improved with carbamylated darbepoetin. These improvements were comparable with CEPO and erythropoietin.	Infarct areas were significantly decreased with carbamylated darbepoetin treatment. These results were comparable with CEPO and erythropoietin.
Mann et al. J. Exp. Neurol 2008[138].	Male SD rats 320 – 340g	**Spinal cord injury:** T9/T10 Contustion with the Ohio State University (OSU) Impactor (200-260 kdyn)	Darbepoetin (10μg/kg) given by IV injection. Or Recombinant human erythropoietin (rhEPO) (5000 IU/kg)	Single injection given 1h post injury.	1. Vehicle control (n=11) 2. EPO (n=12) 3. Darbepoetin (n=11)	The BBB, hindpaw sensory, footfall, and footprint analyses were performed. Treatment with either EPO or Darbepoetin did not yield any significant improvements in neurological recovery.	Histologic analysis (EC stain) of white and grey matter did not show any significant neuroprotective effects associated with either EPO or Darbepoetin.

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 1/6)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Spinal Cord Injury (SCI)							
Mann et al, 2008 Chapter 3 of this thesis.	Male SD rats (320-340g)	SCI: Ohio State University (OSU) Impactor contusion at T9/T10. (1.5mm displacement, 200-260 kdyn)	Atorvastatin (AT) at 5mg/kg/day or simvastatin at 20mg/kg/day (SV) – both delivered via oral gavage (in saline).	Treatment with statins started 1hr after injury and continued once a day for one week.	1. AV (5mg/kg/day) (n=4) 2. SV (20mg/kg/day) (n=5) 3. Vehicle (n=9)	Only Simvastatin improved neurologic outcomes (BBB scores and BBB subscores were significantly improved at 42 days post injury)	Only simvastatin increased the amount of spinal cord white matter that was spared at the injury site. Furthermore, simvastatin reduced macrophage accumulation and increased GFAP expression at the injury site (42 days post injury).
Mann et al, 2008 Chapter 4 of this thesis.	Male SD rats (290-320g)	SCI: Ohio State University (OSU) Impactor contusion at T9/T10. (1.5mm displacement, 200-260 kdyn)	Simvastatin (SV) at 20mg/kg/day and 5mg/kg/day. SV was chemically activated and injected subcutaneously.	SV (20mg/kg) treatment started 1hr after injury and continued for 3 days. 4 days after injury SV dosage was reduced to 5mg/kg and continued for the duration of the experiment (6weeks)	1. SV (n=11) 2 Vehicle (n=12)	No improvement of neurologic recovery was observed with the long term SV treatment regimen.	Although simvastatin significantly reduced macrophage accumulation within the spinal cord (3days post injury), no effects were observed on white matter or grey matter sparing 42 days after spinal cord injury.
Pannu et al. J Neurochem, 2007[8].	Female SD rats (225-250g)	SCI: Weight drop contusion at T12 (40g cm force)	Atorvastain (AT) delivered by oral gavage (5mg/kg). Saline was used as the vehicle treatment.	Started 2h, 4h, or 6h post injury, continued until animals sacrificed (day42 was the latest end-point)	Behavioral Analysis 1. Saline (n=9/group) 2. AT (n=9/group) Histology/biochemistry 1. Saline (n=3 /group) 2. AT (n=3 /group)	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.	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&E stain), reduces neutrophil and macrophage infiltration, MMP 9 activity and expression, and Evans Blue extravasation. 5days post injury, AT reduced GFAP expression (mRNA)

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 2/6)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Pannu et al. J Neurosci Res, 2005[7].	Female SD rats (225-250g)	SCI: Weight drop contusion at T12 (30g cm force)	Atorvastatin (AT) 5m/kg/day in saline, orally until death.	Started 7 days before SCI, given until animals sacrificed (Day 15 was the latest end-point).	Behavioral Analysis 1. Saline (n=9/group) 2. AT (n=9/group) Histology/biochemistry 1. Saline (n=3 /group) 2. AT (n=3 /group)	AT promotes significant locomotor recovery. At 15 days post-SCI, BBB scores were ~19 in AT treated and ~9 in controls.	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.
Ischemic Stroke							
Chen et al., Ann Neurol, 2003[142].	Male Wistar rats (270-300g)	Stroke: Middle cerebral artery occlusion (for 2 hours)	Atorvastatin, AV (1mg/kg, 3mg/kg, 8mg/kg) or simvastatin, SV (1mg/kg) were delivered by oral gavage. Saline was used as a vehicle to deliver drugs.	Treatment was started 24 hours after stroke and continued for 7 days, once a day.	1. AV 1mg/kg/day (n=12) 2. AV 3mg/kg/day (n=12) 3. AV 8mg/kg/day (n=12) 4. Control (n=12) 5. SV 1mg/kg/day (n=8)	Atorvastatin 1-3mg/kg/day) improved functional recovery. Simvastatin (1mg/kg/day) was just as effective as atorvstatin at improving functional recovery. Treatment with AV at 8mg/kg/day did not significantly improve recovery.	14 days after injury, animals were sacrificed. Lesion volumes of treated animals were not significantly different from those of treated with control. Statin treatment resulted in increased VEGF expression, cGMP (increased eNOS activity), angiogenisis, neurogenesis, and synaptophysin. These results suggest that statins increase plasticity after ischemic injury to the brain.
Chen et al. J Cereb Blood Flow Metab, 2005[143].	Male C57BL/6 J mice (24-28g)	Stroke: Middle cerebral artery occlusion	Atorvastatin (10mg/kg), AV, was dissolved in methanol/saline and injected subcutaneously.	Treatment was started 24 hours after stroke and continued once a day for 7 or 14 days.	14 day survival 1. Saline control (n=12) 2. AV 10mg/kg/day (n= 11) 7 day survival 1. Saline Control (n=10) 2. AV 10mg/kg/day (n=10)	AV treated Mice showed significantly improved neurologic recovery at both 7 and 14 days after stroke.	There was no differences in brain lesion volume between treated and control animals. AV treatment, however, resulted in increased neurogenesis, neuronal plasticity, VEGF expression, VEGFr expression, and BDNF expression. Furthermore, there was increased endothelial cell proliferation and neuronal migration. These effects suggest increased brain plasticity and this is thought to be responsible for the observed increase in neurologic recovery with AV treatment.

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 3/6)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Ischemic Stroke							
Endres et al. PNAS, 1998[144].	129/SVEvTacBR mice, C57BL/6NCrlBR mice, eNOS deficient mice (18-22g)	Stroke: Middle cerebral artery occlusion for 2 hours - followed by reperfusion.	Simvastatin, SV, or Lovastatin, LV, (0.2 - 20mg/kg/day) injected subcutaneously. (Drugs had to be chemically activated before use)	Treatment began 3 or 14 days before stroke. Animals received drugs once a day until 24 hours or 3 days after stroke.	24 hour survival 1. 14 days SV or LV treatment 2. 3 days SV treatment 3. Control 3 day survival 1. 14 days SV treatment 2. 3 days SV treatment 3. Control	Functional deficits were reduced in concentration dependent manner at both 24 hours and 3 days after stroke. 3 day pretreatment had a smaller effect than 14 day pretreatment. LV had a smaller effect than SV after 14 days of pretreatment.	Infarct volumes were also reduced in concentration dependent manner, with simvastatin having the greatest effect. Statins increase eNOS expression and cerebral blood flow after injury. eNOS deficient mice failed to show any improvements after statin treatment, suggesting that the observed neuroprotective effects of these drugs are eNOS dependent.
Sironi et al., Arterioscler Throm b Vasc Biol, 2003[145].	Male SD Rats (200-250g)	Stroke: Middle cerebral artery occlusion	Simvastatin, SV, (chemically activated before use). Atorvastatin, AV. Drugs delivered by subcutaneous injection at 0.2 – 20mg/kg/day)	Animals were treated with simvastatin: once a day for 3 days before injury, or 3 and 25 hours after injury.	Pretreatment for 3 days before injury 1. SV (20mg/kg/day) (n=12) 2. Vehicle (n=12) Post ischemic treatment at 3 and 25 hours after injury 3. Vehicle (n=12) 4. SV 20mg/kg (n=12) 5. SV 2mg/kg (n=6) 6. SV 0.2mg/kg (n=6) 7. AV 10mg/kg (n=6)	N/A	Brain infarct volume (MRI) was decreased with SV or AV treatment at 48 hours post ischemia in a dose dependent manner. A neuroprotective effect was reported in both pretreatment and post treatment paradigms. Authors only go as far as to say that AV (10mg/kg) had a smaller effect than 20mg/kg SV. eNOS immunoreactivity increased in SV treated rats.
Baldui ni et al., Stroke, 2001[146].	Newborn Male SD rats (post natal day 1 pups)	Stroke: Ligation of right common carotid artery on post natal day 7, followed by hypoxia.	Simvastatin, SV, (chemically activated before use), delivered by subcutaneous injection at 20mg/kg/day)	Daily injections of SV from post natal day 1 to day 7.	1. Ischemia + vehicle treatment 2. Ischemia + SV treatment 3. Sham injury + Vehicle 4. Sham injury + SV	SV treated rats had significantly better scores on several neurologic tests after ischemic injury.	Histological improvements were observed in SV treated rats - brain damage after ischemia was reduced with SV.

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 4/6)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Ischemic Stroke							
Yrjanheikki et al., Brain Res, 2005[147].	Male Long-Evans rats (220-350)	Stroke: permanent middle cerebral artery occlusion.	Atorvastatin, AV, (10mg/kg/day) delivered orally. 1.5% carboxy-methyl-cellulose	Animals treated daily for 14 days before and 7 days after ischemia. Or, animals were treated daily starting 4 hours post ischemia and treatment was continued for 7 days post ischemia.	14 day pre-treatment 1. AV 10mg/kg/day (n=10) 2. Vehicle (n=10 Post-treatment (7 days) 3. AV 10mg/kg/day (n=10) 4. Vehicle (n=10	AV pre-treated animals had significantly better neurologic scores. Post treatment had no effect on functional recovery.	Only AV pre treated rats had reduced infarct volumes at 7 and 21 days post ischemia. AV did not alter cerebral hemodynamic parameters. Mechanism of AV's action is unclear.
McGirt et al., Stroke, 2002[148].	Male mice, C57BL/6J	Subarachnoid hemorrhage: perforate the right anterior cerebral artery.	Simvastatin (SV), chemically activated and injected subcutaneously (20mg/kg/day)	Animals pre-treated for 14 days (prior to hemorrhage). Or treated post-injury at 30mins, 24hr, and 48hr after injury.	14 day pretreatment 1. SV 20mg/kg/day (n=34) 2. Vehicle (n=36) 3 day post injury treatment 3. SV 20mg/kg/day (n=37) 4. Vehicle (n=38)	Pretreatment and posttreatment with SV reduced neurological deficits (at 72h post injury).	Pretreatment and posttreatment with SV reduced cerebral vasospasm (diameter of injured artery). There was a significant increase in eNOS in response to pretreatment, but after postreatment this increase was not significant.
Amin-Hanjani et al., Stroke, 2001[149].	Male WT mice and eNOS deficient mice	Stroke: middle cerebral artery occlusion for 2hrs followed by reperfusion.	Mevastatin (MV), chemically activated and injected subcutaneously via miniosmotic pumps. (2 or 20mg/kg/day)	Animals pre-treated once a day for 7,14,28 days 14 days (prior to hemorrhage).	Animals treated for 7, 14, or 28 days (n=9 to 12) 1. MV, 2mg/kg/day pretreatment 2. MV, 20mg/kg/day pretreatment 3. Vehicle	24h after injury, neurologic deficit was reduced with MV treatment in a dose dependent manner.	24h after injury, infarct volume was reduced with MV treatment in a dose dependent manner. MV treatment resulted in increased cerebral blood flow and eNOS expression (mRNA).
Baldunini et al., Stroke, 2003[150].	Post natal day 7 (P7) SD Rats	Stroke: On Post natal day 7, the right common carotid artery was ligated for 3 hours, this was followed by hypoxia.	Simvastatin (SV), chemically activated and injected subcutaneously (20mg/kg/day)	Animals were Pre-treated from Post natal day 1 (P1) to P7. OR Animals were treated from P4-P11. Or P7-P14 (post-treatment)	1. SV P1-P7 (20mg/kg/day) 2. SV P4-P11 (20mg/kg/day) 3. SV P7-P14 (20mg/kg/day) 4. Vehicle P1-P7 5. Vehicle P4-P11 6. Vehicle P7-P14 n=7 for each group	Groups that were treated with SV from P1-P7 and P4-P11 had better functional recovery. Delayed post ischemic (P7-P14) treatment had no effect.	Groups that were treated with SV from P1-P7 and P4-P11 showed decreased brain damage. Pretreatment with SV resulted in decreased expression of TNF-alpha, IL-1beta. However, SV had no effect on eNOS expression.

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 5/6)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Traumatic Brain Injury (TBI)							
Lu et al., J Neurotrauma, 2004[151].	Male Wistar Rats (300-400)	TBI: A pneumatic device was used to deliver an impact to the left (sensimotor) cortex	Atorvastatin (AV) delivered orally (1mg/kg/day) in saline.	Treatment started 1 day after TBI. Treatment was then continued daily for 7 days.	1. AV 1mg/kg/day (n=10) 2. Saline – vehicle-treatment (n=10)	AV treatment for 7 days, starting one day after TBI, significantly increased functional recovery.	AV treatment improved histological outcomes (14 days post injury) Furthermore, neuronal survival, synaptogenesis, and angiogenesis were increased.
Wang et al., Exp Neurol, 2007[152].	Male mice, C57BI/6 J (10-12 weeks)	TBI: closed cranial trauma delivered by a pneumatic impactor.	Atorvastatin (AV) or simvastatin (SV) were injected subcutaneously. SV was chemically activated before delivery. Both drugs delivered at 20mg/kg/day.	Animals were pretreated with for 14 days prior to injury and then for 3 days after TBI. OR Animals were only post-treated for 3 days after TBI.	Pre treatment (14 days pre injury + 3 days post injury) 1. SV (20mg/kg/day) 2. AV (20mg/kg/day) 3. Vehicle Post injury treatment only (3 days post injury) 1. SV (20mg/kg/day) 2. AV (20mg/kg/day) 3. Vehicle	AV and SV both reduce functional deficits. Pretreatment or post injury treatment paradigms of either drug produced increased neurologic recovery. No difference was observed between drugs and they seem to be equal in their neuroprotective effect. AV was chosen for most experiments because of its higher plasma half-life.	AV and SV both reduce histological deficits. Statin treatment also resulted in the reduction of degenerating hippocampal neurons, the suppression of inflammatory cytokines, and improved cerbral hemodynamics (blood flow).
Lu et al., J Neurotrauma, 2007[153].	Wistar rats, Male (300-400g)	TBI: Direct impact, with a pneumatic piston, to the (left) cortex.	Simvastatin (SV) or atorvastatin (AV) was delivered by oral gavage at 1mg/kg/day (in saline).	Treatment was started 1 day after injury and continued for 14 days.	1. AV (1mg/kg/day) (n=10) 2. SV (1mg/kg/day) (n=10) 3. Vehicle (n=10)	Statin treatment improved neurologic recovery. This was observed through improved spatial learning tasks after drug treatment. Simvastatin was superior to AV at achieving these effects at the same dose.	Statin treatment reduces neuronal loss, increases neurogenesis, improves angiogenesis. Simvastatin, at the same dose as ATV, was superior at achieving these effects.

Table 1.5.3 The evaluation of statins for different forms of neurological injury (Page 6/6)							
Authors	**Animal Model**	**Injury Model**	**Intervention**	**Intervention Timing**	**Experimental Groups**	**Behavioral Outcome**	**Histologic / Biochemical/ Physiologic Outcome**
Multiple Sclerosis (MS)							
Paintlia et al., Faseb J, 2005[154].	Female Lewis Rats (225-300)	MS: Experimental Autoimmune Encephalitis (EAE) (Sub-cutaneous injection of MBP in CFA)	Lovastatin (LV) was delivered via intra-peritoneal injection at 2mg/kg/day (in TX-100/PBS)	Treatment with LV started immediately after MBP injection and before the onset of EAE. LV treatment continued once a day for the duration of expt.	1. EAE + LV (2mg/kg/day) 2. EAE + vehicle 3. Sham + LV (2mg/kg/day) 4. Sham + Vehicle	LV treated animals had lower EAE scores and better neurological function.	LV treatment resulted in decreased demyelination and inflammatory cell infiltration in the white matter of the spinal cord. LV also increased survival and differentiation of oligodendrocytes.
Youssef et al., Nature, 2002[123].	Female SJL/J, B10.PL and C57BI/6J mice (8-12 weeks old)	MS: Experimental Autoimmune Encephalitis (EAE) (subcutaneous injection of PLP, MOG, or MBP in CFA)	Atorvastatin (AV) was delivered orally at 1mg/kg/day or 10mg/kg/day (in PBS)	Once a day starting at the first clinical onset of EAE or after the acute EAE phase was established.	There were numerous treatment arms, main groups were: 1. EAE + AV starting at clinical onset (10mg/kg/day) 2. EAE + AV starting at clinical onset (1mg/kg/day) 5. EAE + vehicle starting at clinical onset 3. EAE + AV starting during acute EAE (10mg/kg/day) 4. EAE + AV during acute EAE (1mg/kg/day) 5. EAE + during acute EAE vehicle	When delivered at clinical onset or after acute EAE was established, both a 1mg/kg/day or 10mg/kg/day treatment of AV reduced symptoms of EAE. AV also prevented exacerbations of a chronic relapsing form of EAE.	AV increased the expression of Th2 cytokines (IL-4, IL-5, TGF-β, and IL-10). On the other hand AV reduced the expression of Th1 cytokines (IL-2, IL-12, IFN-γ, and TNF-α). AV also reduced the expression of MHC class II proteins in the CNS. Therefore, immunomodulation and the reduction of autoimmune mediated damage are believed to be the mechanism behind AV's beneficial effects for EAE.

Table 1.5.4 The evaluation of minocycline for spinal cord injury (Page 1/3)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Wells et al., Brain, 2003[10].	Male CD-1 Mice 3 months old	T3/T4 Extradural compression using a modified aneurysm clip (8g force for 1 min)	Minocycline (Mino) (Sigma) at 50mg/kg or 25mg/kg given by IP injection (in saline).	1h after injury (50mg/kg) and then once a day for 5 days (25mg/kg).	1. Saline (n=15) 2. Minocycline (n=18) 2nd set of experiments 1. Minocycline (n=10) 2. Vehicle (n=10) 3. Vehicle pH 5 (n=10 4. MPSS 30mg/kg) (n=10)	BBB scores were significantly higher in the minocycline treated group starting 3 days post injury (to day 28) and inclined plane scores were improved starting 21 days after injury (to day 28). MPSS had no effect and neither did a pH 5 vehicle solution, hence it is not the acidic solution that is mediating the observed effects of Minocycline.	The size of the lesion at the injury site was quantified and determined to be significantly smaller in the Mino treated group.
Lee et al., J. Neurotrauma, 2003[155].	Male SD Rats 230-250 g	T9/T10 Weight drop injury (12.5g-cm)	Minocycline (Sigma), at 90mg/kg or 45mg/kg, was given by IP injection (in PBS).	First dose (90mg/kg) was given immediately after injury, then 2 more were given (45mg/kg) every 12h.	Histology 1. PBS (n=3) 2. Minocycline (n=3) Behavior 1. PBS (n=13) 2. Minocycline (n=13)	Animals treated with Mino had significantly better locomotor recovery, assessed by the BBB scale. Improvements were significant starting 24 days after injury and remained significant until day 38 (~18-Mino vs ~15-PBS). Authors also state that pretreatment or the extension of minocycline treatment for 10 days had negative side effects, these treatment paradigms were not as beneficial neurologically.	Lesion size was significantly less in the Mino group at day 28 and day 38. Caspase-3 activity and the number of apoptotic cells (TUNEL) were significantly reduced 24h after injury. IL-10 was significantly increased (6hr) and TNF-α was significantly reduced (6 and 12h) in spinal cord tissue after injury.
Zang et al., J. Neurotrauma, 2003[156].	C57BL/6 J mice (male and Female) (6 weeks old)	T12 – A series of lesions performed on each animal: Bilateral dorsal hemisection + Right hemisection + Bilateral ventral hemisection.	Minocycline given by IP injection at 10mg/kg. Vehicle (1% albumin in 0.1M PBS)	Given 2h after injury and continued at 3 times a week.	1. Vehicle (n=10) 2. Minocycline (n=10)	Mino had no effect on neurologic recovery (rotorod, Bar grab, bar walk, platform hang). Leukemia inhibitory factor (LIF) was also assessed in this study and was reported to improve neurologic recovery in this model.	Mino treatment resulted in no significant sparing of myelinated axons.

Table 1.5.4 The evaluation of minocycline for spinal cord injury (Page 2/3)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
McPhail et al, Eur. J. Neurosci., 2004[157].	Long Evens rats and Long Evens Shaker rats (failure to produce functional myelin in adulthood).	T10 dorsal column crush (fine forceps)	Minocycline (100mg/kg or 25mg/kg) (Sigma) daily IP injections.	100mg/kg/day pre injury and 25mg/kg/day post injury. Killed 7days post injury.	1. LE (n=6) 2. LE + Mino (n=4) 3. LEShaker (n=5) 4. LEShaker + zymosan (pro inflammatory) (n=4)	None	Axons did not retract from lesion site in shaker rats as they do in normal rats. Shaker rats had reduced macrophage response at injury site and reduced schwann cell response. Increasing the macrophage response had no effect in shaker rats. Minocycline reduces macrophage, astroglial, and the schwann cell response, and prevents axon dieback.
Teng et al., PNAS, 2004[158].	Female SD rats (280–330g)	T10 NYU weight drop contusion (12.5g-cm).	Minocycline (90mg/kg or 45mg/kg) (Sigma) in normal saline (heated), injected IP	Animals were treated with 90mg/kg 1h after injury and 45mg/kg every 12 afterwards, for 5 days.	Biochemistry 1. Saline (n=3) 2. Minocycline (n=3) Histology 1. Saline (n=8-10) 2. Minocycline (n=8-10) Behavior 1. PBS (n=10) 2. Minocycline (n=10)	Mino had no effect on animal health and weight; there was no tissue necrosis observed in the peritoneum. Mino significantly improved motor recovery on the incline plane test and on the BBB locomotor scale. BBB scores were ~13-Saline vs ~17-Mino at day 28 post injury.	Mino had no effect on lesion volume, however it significantly preserved both white matter and ventral horn neurons (4 weeks post injury). GFAP immunoreactivity was significantly decreased and CNPase immunoreactivity (oligodendrocyte preservation) was increased with Mino treatment. Mino (90mg/kg and 180mg/kg) also prevents the release of Cytocrhome C from mitochondria 4h after injury.
Stirling et al., J. Neurosci, 2004[9].	Wistar Rats "Adult"	C7 Dorsal column transection.	Minocycline (50mg/kg) (Apotex), dissolved in saline injected IP.	Mino treatment (50mg/kg) began 30mins after injury and was continued for 2days.	1. Saline 2. Mino	Mino significantly improved interlimb coordination and reduced hindlimb angle of rotation 7 and 14 days after injury.	Mino reduced apoptosis of glial cells (microglia and oligodendrocytes) 7 days post injury. ED1+ microglial/macrophage density in the CST and the AST was reduced 7 days post injury. CST dieback and lesion area were significantly reduced 7 and 14 days post injury.

Table 1.5.4 The evaluation of minocycline for spinal cord injury (Page 3/3)

Authors	Animal Model	Injury Model	Intervention	Intervention Timing	Experimental Groups	Behavioral Outcome	Histologic / Biochemical/ Physiologic Outcome
Festoff et al., J Neuroc hem, 2006[159] .	Hooded and SD rats 300-325g	T9 NYU weight drop contusion (25g-cm)	Minocycline (30mg/kg or 90mg/kg) (Sigma) injected IP.	Multiple injections of 30mg/kg at 30mins, 1h and 24h after injury. Single injection of 90mg/kg at 30min or 1h or 24h after injury.	1. Mino 30mg/kg 30min + 1h + 24h after injury (n=4) 2. Mino 90mg/kg 30min after injury (n=4) 3. Mino 90mg/kg 1h after injury (n=4) 4. Mino 90mg/kg 24h after injury (n=2) 5. Control (tetracycline) 30mg/kg 1h	At day 28, BBB scores were remarkably improved in the multiple 30mg/kg Mino dose group. At day 28, 90mg/kg of Mino improved BBB scores; delaying the treatment to 24h does not seem affect the overall effect observed. Note: sample sizes were very small.	Mino reduces tissue cavity formation, active caspase 3 activity, TNF-alpha expression, and microglial activation.
Yune et al., J. Neuros ci, 2007[160] .	Adult SD rats 250 - 300g	T9/T10 Weight drop (NYU) contusion (25g-cm)	Minocycline (90mg/kg or 45mg/kg) (Sigma) dissolved in PBS injected IP.	90mg/kg administered immediately after injury (biochemistry) or 2h after injury (histology and behavior), then administered 45mg/kg every 12h for 3 days after initial dose.	Behavior n=20/group Histology n=3/group Biochemistry n=6/group 1. Mino 2. Vehicle 3. MPSS	Mino treatment significantly improved BBB locotmotor scores (starting day20 to day 35 post injury), inclined plane test (starting 1 week to 4weeks after SCI), and grid error test (5weeks post SCI). Delaying the Mino treatment 2h post SCI seemed to have no effect on its efficacy.	Minocycline inhibits proNGF expression after injury (mRNA + Protein) 5 days post injury. p38 MAPK and MAPKAPK-2 activation (in microglia) was reduced after Mino treatment (day 3 and 5 post injury). Mino also inhibits the expression of p75 in oligodendrocytes (NT receptor) (mRNA and protein) 5 days post injury. Mino also prevents the activation of RhoA 3days and 5days post SCI. Mino reduced the number of apopotic oligodendrocytes (5days after injury). 38 days post SCI, axon loss was attenuated with Mino treatment.
Mann et al, 2008 Chapte r 3 of this thesis.	Male SD rats (320-340g)	SCI: Ohio State University (OSU) Impactor contusion at T9/T10. (1.5mm displacem ent)	Minocyline (Sigma) delivered by IP injection at 90mg/kg and 45mg/kg.	First dose of 90mg/kg was given 1h after injury. This was then followed by a 45mg/kg dose every 12h for 3 days.	1. Minocycline (n=11) 2. Vehicle (n=12)	Minocycline treatment had no influence on behavioral recovery, assessed by the BBB locomotor test and catwalk gait analysis system.	Minocycline treatment had no influence on histological outcomes (secondary pathology) after spinal cord injury.

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CHAPTER 2

The comparison of intravenously delivered darbepoetin and erythropoietin in their ability to improve outcomes after spinal cord injury[2]

[2] A version of this chapter has been published. Cody Mann, Jae H.T. Lee, Jie Liu, Anthea M.T. Stammers, Hong-Moon Sohn, Wolfram Tetzlaff, Brian K. Kwon. Delayed treatment of spinal cord injury with erythropoietin or darbepoetin – A lack of neuroprotective efficacy in a contusion model of cord injury (2008). Experimental Neurology, 211:34-40.

2.1 INTRODUCTION

The use of recombinant human erythropoietin to stimulate red blood cell production in the bone marrow and treat anemia is a long-standing clinical practice. Over the past decade, erythropoietin has been found to have significant neuroprotective properties[1,2]. Erythropoietin and its receptor have been identified in neurons and glial cells within the central nervous system[3]. It has recently been reported that following a compressive spinal cord injury, the expression of erythropoietin and its receptors within the neurons, vascular endothelium, and glial cells of the injured area increases, and that this expression in the vascular endothelium and glial cells is maintained for week[4]. A number of independent laboratories have evaluated erythropoietin in thoracic spinal cord contusion or compression injury models, and have reported various neuroprotective effects, including reduced secondary damage, lipid peroxidation, and programmed cell death, and improved behavioral outcomes (Table 2.5.1)[5-12].

Darbepoetin, as the name suggests, is a derivative of erythropoietin. By the addition of oligosaccharides with sialic acid residues onto the native erythropoietin molecule, the half-life of darbepoetin is extended and its biologic activity increased[13]. In pharmacokinetic studies, darbepoetin was found to have a much greater terminal half-life than erythropoietin (25.3 vs 8.5 hours)[14]. Much less is known about darbepoetin's neuroprotective properties as compared to erythropoietin. In a rat model of cerebral ischemia, darbepoetin treated animals had significantly improved neurological scores, and reduced infarct volume within the brain[13,15]. A more recent study found that carbamylated darbepoetin was comparable to erythropoietin in reducing infarct volume and improving sensorimotor function after middle cerebral artery occlusion in rats[16].

A study of the effects of darbepoetin in spinal cord injury and its relative efficacy compared to erythropoietin in this setting has not been previously reported. Darbepoetin's increased half-life and biologic activity provide compelling rationale to evaluate it in this setting. The previously demonstrated neuroprotective properties of erythropoietin have stimulated interest in translating this widely used drug into human spinal cord injury trials[4] and has already led to the initiation of a human clinical trials in stroke[17] and in extradural spinal cord compression from metastatic disease[18]. However, acknowledging the enormous time, effort, and resources required to conduct an acute human spinal cord injury neuroprotection trial, it would

seem rational to at least first explore the relative efficacy of darbepoetin against erythropoetin in a preclinical model, given the former's potential pharmacokinetic advantages.

Therefore, ***I hypothesize that darbepoetin will improve neurologic and histologic outcomes with greater efficacy than erythropoietin in a clinically relevant experimental model of spinal cord injury***. The dosing regimens that will be used are based on previous studies that have been performed by others and have demonstrated them as neuroprotective. One bolus of erythropoietin will be delivered intravenously at 5000 IU/kg, one hour after injury - a dose that has been effective in experimental spinal cord injury[5,6,8-10,12]. Although darbepoetin has never been evaluated in experimental spinal cord injury, a 10 μg/kg intraperitoneal dose has been efficacious in a rodent model of brain ischemia[15]. Hence, in this study, a dose of 10 μg/kg will be delivered one hour after injury.

2.2 MATERIALS AND METHODS

Animal Model and Surgical Procedures

40 male Sprague-Dawley rats weighing 320-340 gm were anesthetized with an intraperitoneal 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 a dorsal midline incision was made over the mid-thoracic spine to expose the posterior spinal elements at T9-10. A laminectomy was performed, and the bases of the adjacent spinous processes were secured with modified Allis clamps which then held the animal secure within a custom frame. With half of the animal's weight suspended, the animal was positioned under the Ohio State University (OSU) Impactor, and the impactor tip was gently lowered to apply a preload force of 0.2 kdyne onto the dura. The impactor was then triggered to deliver a 1.5 mm displacement injury at 300 mm/s. Animals were excluded if the peak force fell outside the range of 200-260 kdynes. The dorsal wound was then closed. (For detailed methodology on injury model and surgery, see appendix 6.1.1)

One hour later, an incision on the ventral aspect of the neck was made and the left jugular vein was cannulated. Animals were randomized to receive a single bolus intravenous injection of either 1. Erythropoietin, 5000 IU/kg (Eprex®, Janssen-Ortho Inc., Toronto, Ontario, Canada), 2. Darbepoetin, 10 μg/kg (Aranesp®, Amgen, Thousand Orks, California, USA), or 3. Saline, 1 cc. Animal temperature was monitored and maintained at 37° Celsius in an incubator during the

acute postoperative period to avoid the potential neuroprotection afforded by hypothermia. Manual bladder expression was performed 3 times per day until reflexive voiding returned. The animals received a subcutaneous injection of buprenorphrine (0.02 mg/kg, Temgesic®, Reckitt Benkiser HealthcareLtd., UK) just prior to their surgery and then again on post-operative days 1 and 2. All animals' surgeries and care were conducted in accordance to UBC Animal Care guidelines.

Behavioral Outcome Assessment

For all behavioral outcome assessments, baseline scores were first established by evaluating the animals prior to being injured. All evaluations post-injury were performed by individuals blinded to treatment group.

Open-field locomotor testing was performed by two blinded examiners on days 2, 7, 14, 28, 35, and 42 days post-injury using the Basso, Bresnahan, and Beattie (BBB) locomotor scale and subscore[19,20]. All open-field locomotor testing was performed under the same conditions and at the same time period on each scheduled testing date. (For detailed methodology on behavioral assessment, see appendix 6.1.2)

At 42 days post-injury, the animals were videotaped as they walked over a horizontally laid ladder with unevenly spaced rungs. Subsequent analysis determined the number of times the hindlimb slipped between the rungs, this was counted as a ("footfalls"). As a final measurement of functional outcome, the number of footfalls was divided by the total number of steps taken to produce a "percentage of error" value. Also, the width between the hindlimbs ("base of support") was measured off of high-definition video footage of the animals walking.

The animals underwent hindpaw pinprick sensory threshold testing at 42 days post-injury using a von Frey monofilament device (Semmes-Weinstein monofilament, Stoelting Co., Wood Dale, IL) to evaluate whether mechanical allodynia was present. In brief, the animal was placed on an elevated grid and observed for 5 to 10 minutes to ensure that it was calm. When both hindpaws were evenly resting upon the rungs of the grid, the monofilament was pressed into the center of the volar surface of the hindpaw. The mechanical force at which hindlimb withdrawal occurred and the time from stimulus application to hindlimb withdrawal were recorded.

Anatomic Outcome Assessment

At 42 days post-injury, all animals were sacrificed with a lethal injection of Pentobarbital Sodium (107 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and then

perfused with phosphate buffered saline followed by fixation with cooled 4% paraformaldehyde. A 15 mm segment of spinal cord centered around the injury site was harvested, post-fixed overnight in 4% paraformaldehyde, cryoprotected in increasing doses of sucrose, and then snap frozen over dry ice. The cords were sectioned axially at 20 μm thickness for histological analysis of the injury site. Sections 200 μm apart were stained with Eriochrome Cyanine (EC) as per described by Rabchevsky et al[21], counter-stained with Neutral Red, then imaged on a Zeiss Axioskop microscope at 5x objective. Regions of spared white and grey matter were manually traced and then quantified using SigmaScan Pro version 5.0.0 (Systat Software Inc.).

Statistical analysis

All statistical analyses were performed using SPSS 10.0 for Windows. Differences among the treatment groups were tested using a one or two-way analysis of variance (ANOVA) and the least significant difference (LSD) multiple comparisons test when warranted. For sensory and footfall test, data were compared between groups using a Student's t-test. Differences with a P value less than 0.05 were considered statistically significant.

2.3 RESULTS

A total of 34 animals were randomized to either erythropoietin (n=12), darbepoetin (n=11), or saline control (n=11). The mean displacement and peak force were very similar amongst the three groups (Table 2.5.2), indicating that the biomechanical characteristics of the thoracic contusion injury were comparable across the groups.

Following spinal cord contusion at T9/10, no improvement of locomotor function was observed in animals treated with either erythropoietin or darbepoetin as compared to saline controls in the open field locomotor functional test. The mean BBB scores at 42 days post-injury was 11.7 ± 0.4, 11.6 ± 0.3, and 11.3 ± 0.2 for animals treated with erythropoietin, darbepoetin, and saline, respectively ($p > 0.05$, Two-Way ANOVA multiple comparison). At no time point during the post-operative period was there any statistically significant difference between the groups to suggest a change in the rate of recovery (Figure 2.5.1 A). Similarly, the BBB subcores revealed no significant differences between the groups, although it was slightly improved in both groups treated with erythropoietin and darbepoetin four weeks after spinal cord injury as compared to the saline controls (Figure 2.5.1 B).

For gait testing on the unevenly spaced rungs, all animals demonstrated increased numbers of stepping errors post-injury, but there were no significant differences between the erythropoietin, darbepoetin, and saline controls at 42 days post-injury. The percentage of stepping errors of hindlimbs was 21.3 ± 3.8, 21.8 ± 3.7, and 26.8 ± 3.1 for animals treated with erythropoietin, darbepoetin, and saline, respectively ($P > 0.05$, Student-t Test) (Figure 2.5.2). The base support of hindlimbs was 54.03 ± 4.3, 52.7 ± 3.0, and 53.8 ± 3.4 mm for rats treated with erythropoietin, darbepoetin, and saline, respectively. Similarly, there was an increase in the width of the hindlimb base of support in all animals after injury, but no differences amongst the erythropoietin, darbepoetin, and saline controls ($P > 0.05$, Student-t Test) (Figure 2.5.3). Specifically, neither erythropoietin nor darbepoetin reduced the number of footfalls or reduced the hindlimb base of support as compared to the control animals.

For mechanical sensory testing, the force at which hindpaw withdrawal occurred increased in all animals post-injury (reflective of impaired sensation). The maximal force recorded when hindpaw withdrawal was 36.5 ± 2.5, 35.2 ± 2.1, and 37.2 ± 2.8 grams for rats treated with erythropoietin, darbepoetin, and saline, respectively, but there was again no significant differences between the erythropoietin, darbepoetin, and saline controls ($p > 0.05$,

Student t-Test) (Figure 2.5.4). In particular, there was no evidence that erythropoietin or darbepoetin influenced sensory recovery, or the development of mechanical allodynia.

In agreement with the outcome of behavioral tests, histological analysis did not indicate any increased preservation of the contused spinal tissue by treatment of either erythropoietin or darbepoetin, compared to the saline controls. Six weeks after SCI, an evident cavity was developed at lesion area and only a thin rim of spared neural tissue was left at the lesion epicenter in all animals (Figure 2.5.5 A). Based on histological staining by Eriochrome Cyanine, the area of spared white matter at lesion epicenter was 0.46 ± 0.04, 0.47 ± 0.08, and 0.41 ± 0.04 mm^2 for animals treated with erythropoietin, darbepoetin, and saline, respectively (Figure 2.5.5 B). No statistical difference of spared white matter, spanning 3.2 mm distal from the lesion epicenter (in both rostral and caudal directions), was indicated between animals treated with erythropoietin or darbepoetin and saline alone ($p > 0.05$, Two-Way ANOVA multiple comparison). Similarly, no significant increase of spared grey matter was observed in animals treated with either erythropoietin or darbepoetin when compared to the saline controls (Figure 2.5.5 C).

2.4 DISCUSSION

The purpose of this experiment was to compare, in a well-established thoracic spinal cord contusion model, the neuroprotective efficacy of darbepoetin against erythropoietin, the latter having been previously reported to be beneficial in models of spinal cord injury, stroke, and traumatic brain injury. Comparatively little has been reported on the neuroprotective properties of darbepoetin, but its enhanced bioavailability compared to erythropoietin made it seem rational to compare the two in this acute spinal cord injury setting, given the clinical safety of such drugs and their potential for translation into human trials. In summary, this experimental comparison revealed no neuroprotective benefit to either drug as compared to saline controls. Neither behavioral outcome nor sparing of tissue at and around the spinal cord injury site was improved by erythropoietin or darbepoetin.

We were, of course, disappointed by the lack of neuroprotective efficacy of either drug in this experimental setting. We selected a dosage of erythropoietin (5000 IU/kg) that had previously been used by numerous authors (Table 2.5.1) in demonstrating a neuroprotective benefit in thoracic spinal cord injury[5,6,8-10,12]. Furthermore, the 5000 IU/kg dose was found by independent investigators, to be superior to lower doses of the drug, demonstrating a dose-response effect[8,10,12]. It has been our longstanding experience with anesthetic agents that their intraperitoneal injection can result in substantial differences in their rates of absorption (as evidenced by the variation amongst animals in the time and dosage required to achieve a surgical plane of anesthesia). Therefore, we utilized a direct intravenous approach to ensure a more assured and consistent delivery of the drug amongst the animals. While our jugular vein injection requires an additional surgical procedure and is technically more demanding, we can visually confirm that all of the drug has been delivered into the systemic circulation. Gorio et al. compared the IP and IV administration of 5000 IU/kg erythropoietin and reported that serum levels were equivalent by 15 hours post-administration, although it is assumed that the peak serum concentrations are achieved much sooner with the IV injection[9]. While a direct neuroprotective comparison of the 5000 IU/kg dosage was not reported in this study, it was indicated that the intravenous administration of 500 IU/kg was significantly more effective at promoting behavioral recovery when compared to the same dose given intraperitoneally.

The lack of erythropoietin's neuroprotective efficacy in our hands compared to others who used comparable dosages may be attributed to several differences in experimental design. When viewing Table 2.5.1, it is clear that differences exist with respect to the strain, sex, and

weight of the rats utilized. Numerous devices are employed to induce the cord injury, making it almost certain that the biomechanical characteristics of the injury differ amongst studies. The time at which the drug was first administered and how frequently it is administered afterwards differs amongst studies. Of course, if differences of this nature significantly influence a drug's neuroprotective efficacy in the tightly controlled experimental animal setting, then it is hard to envision how such a drug would be effective in the clinical setting where age, race, and injury characteristics, and time to intervention may vary enormously between patients. While our results do not nullify the neuroprotective potential of erythropoietin which has been previously documented by other investigators, they do point to the need for further preclinical investigation to refine the therapeutic approach in spinal cord injury.

As for darbepoetin, much less is known about its neuroprotective properties. At the time that we designed this experiment, the only available report of darbepoetin being evaluated in a neurologic setting was performed by Belayev et al., who studied a 10 μg/kg intraperitoneal dose in a rodent model of brain ischemia induced by middle cerebral artery occlusion[15]. This dosage of darbepoetin was found to significantly reduce total cortical infarct volume compared to vehicle controls, and hence was chosen as the dosage in our experiment. More recently, Villa et al. compared carbamylated, non-erythropoietic forms of erythropoietin and darbepoetin (50 μg/kg each) in a similar rat model of brain ischemia and reported that the efficacy of neuroprotection in reducing cortical infarct area was similar (31% and 28% respectively) between the two drugs[16]. In a non-neurologic application, Johnson et al. also compared the protective effects of erythropoietin and darbepoetin in an animal model of ischemic renal damage, and found that a 25 μg/kg of darbepoetin was comparable to 5000 IU/kg of erythropoietin in terms of reducing renal dysfunction, tissue injury, and apoptotic cell death[22]. The addition of sialic-acid containing oligosaccharides increases the serum half-life of darbepoetin as compared to erythropoietin and thus increases its bioavailability. Interestingly, the affinity for the erythropoietin receptor is actually decreased by these oligosaccharide additions, suggesting that the in vivo bioactivity of erythropoietin and darbepoetin (with respect to erythropoiesis) is determined primarily by serum clearance[13,23]. This prolonged bioavailability, in theory, could be a distinct advantage to a neuroprotective agent administered as a single-injection. While the erythropoeitic effects of erythropoietin and darbepoetin are mediated through erythropoietin receptor homodimers, the non-erythropoeitic neuroprotective effects appear to be mediated through an erythropoietin – common β subunit hetero-receptor

(EpoR-βcR)[24]. The influence of the additional oligosaccharide moieties on darbepoetin on its affinity to this hetero-receptor is unknown.

Developing pharmacologic neuroprotection strategies for spinal cord injury or any other neurologic condition is obviously a very complex process. Lacking in our approach was any form of dose-response study that is clearly a necessary step in substantiating and optimizing a pharmacologic therapy. Data from numerous other authors studying acute contusive thoracic spinal cord injury supported an erythropoietin dosage of 5000 IU/kg. We speculated, therefore, that this would be a reasonable dose to choose for our experiment. Furthermore, we felt that by demonstrating the neuroprotective effect of erythropoietin at this dosage in our spinal cord injury model we would provide additional independent confirmation of this drug's therapeutic potential. We were unfortunately unable to demonstrate this in our experiment. No such data on dosage was available for darbepoetin in previous spinal cord injury studies, and it is evident that our 10 μg/kg dosage that was beneficial in a stroke model[15] was ineffective in this contusive spinal cord injury model. This points to the need for further dose-response studies to better elucidate the potential neuroprotective effect of darbepoetin in spinal cord injury.

In conclusion, our experimental design failed to reveal any neuroprotective benefit to either erythropoietin or darbepoetin in this thoracic contusion spinal cord injury model. We were, as most investigators would be, disappointed in the lack of behavioral or histologic improvement in this study, particularly with erythropoietin. Nonetheless, we feel that such negative results are important to present to the scientific community working in the field of neuroprotection for spinal cord injury. Clearly, further investigation is necessary to refine the therapeutic approach of using erythropoietin or darbepoetin in spinal cord injury.

2.5 FIGURES AND TABLES

Table 2.5.1. Thoracic spinal cord contusion/compression studies that report the neuroprotective effects of erythropoietin

Reference	Rat Strain	Weight (gm)	Injury Model	EPO Dose	Route	Delay	Reported Benefits
Gorio, (2002) PNAS	Sprague Dawley female	240-260	T9 contusion with Modified Univ of Trieste Impactor	5000 IU/kg single dose, 5000 IU/kg x7 days, or 500 u/kg x7 days	IP	1 hour	Improved behavior and tissue sparing at 4 weeks with single 5000 IU/kg dose, which was equivalent to giving it for 7 days; both were better than 500 IU/kg for 7 days.
Kaptanoglu, (2004) Neurosurg Rev	Wistar male	215-260	T7/8 contusion with 50 gm/cm weight drop	100, 1000, 5000 IU/kg single dose	IP	None	5000 IU/kg dose provided the best reduction in lipid peroxidation, and improved unltrastructural neuroprotection at 2 hours post-injury
Gorio, (2005) PNAS	Sprague Dawley	240-260	T9 contusion with Modified Univ of Trieste Impactor	100, 500, or 5000 IU/kg single dose	IP	30 minutes	Best behavioral recovery at 4 weeks with 5000 IU/kg, less with 500 IU/kg
				5000 IU/kg single dose	IP	30 minutes, 24 hrs, 48 hrs	Improved behavior in all groups for the first few days, but only when given at 30 minutes was this improvement sustained over controls at 4 weeks
				100, 500 IU/kg single dose	IV	30 minutes	500 IU/kg dose given IV provided equivalent neuroprotection to 5000 IU/kg given IP, and was better than 500 IU kg given IP.
Boran, (2005) Restor Neurol and Neurosci	Wistar male	180-220	T6/7 contusion with 50 gm/cm weight drop	5000 IU/kg single dose	IP	1 hour	Improved motor function on swimming test at up to two weeks post-injury
Arishima, (2006) Spine	Wistar male	380-500	T8/9 compression with 120 gm weight x 2 minutes	5000 IU/kg x 2 doses (15 minutes and 24 hours post)	IP	15 minutes	Improved tissue protection and decreased TUNEL-positive cells and activated caspase-3 positive cells with EPO at up to 7 days post-injury
Cetin, (2006) Eur Spine J 06	Sprague Dawley	200-300	T3 compression with 0.6 N aneurysm clip x 1 minute	1000 IU/kg single dose	IP	5 minutes	3000 IU/kg dose promoted better behavioral recovery and tissue sparing than 1000 IU/kg at 3 days post-injury. Contrary to Gorio (2005), the combination of 3000 IU/kg with Methylprednisolone provided the best results
				3000 IU/kg x 4 doses (5 minutes then qdaily x 3 days)	IP	5 minutes	
Okutan, (2007) J Clin Neurosci	Wistar female	210-250	T7-9 contusion with 40 gm/cm weight drop	1000 IU/kg single dose	IP	None	At 24 hours post-injury, improved locomotor behavior, reduced myeloperoxidase activity and caspase 3 activation
Vitellaro-Zuccarello, (2007) Neurosci	Sprague Dawley male	240-270	T9 contusion with Modified Univ of Trieste Impactor	5000 IU/kg single dose	IP	30 minutes	Improved locomotor behavior at 4 weeks post-injury and improved tissue sparing, particularly of ventral white matter. Normalization of phosphacan levels with EPO.

Table 2.5.2: Biomechanical parameters of contusion injury.

There were no significant differences amongst the three groups with respect to the peak force of the injury and the displacement of the impactor tip.

Groups	Force (kdynes)	Displacement (mm)	n
Control	229 ± 6.72	1.46 ± 0.03	11
Erythropoietin	227 ± 6.46	1.46 ± 0.03	12
Darbepoetin	223 ± 6.00	1.46 ± 0.03	11

Figure 2.5.1: Open field locomotor function test (BBB scale)

No statistical differences were observed in the BBB scores (A) or subscores (B) amongst the three treatment groups at any time point post-injury.

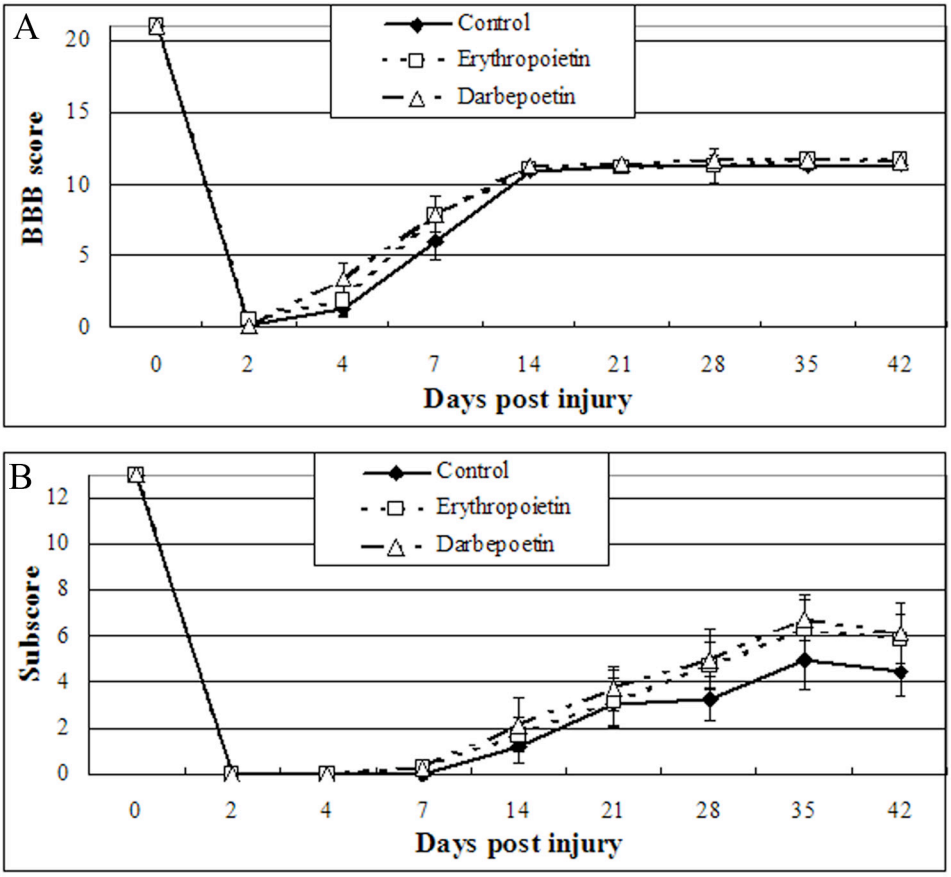


Figure 2.5.2: Horizontal ladder test to measure footfalls.

The number of footfalls (mistakes) made by the animal is divided by the total number of steps. All animal demonstrate increased footfalls 6 weeks post-injury as compared to pre-injury (*, $p<0.01$). No statistically significant improvements were observed in animals treated with erythropoietin or darbepoetin as compared to saline controls.

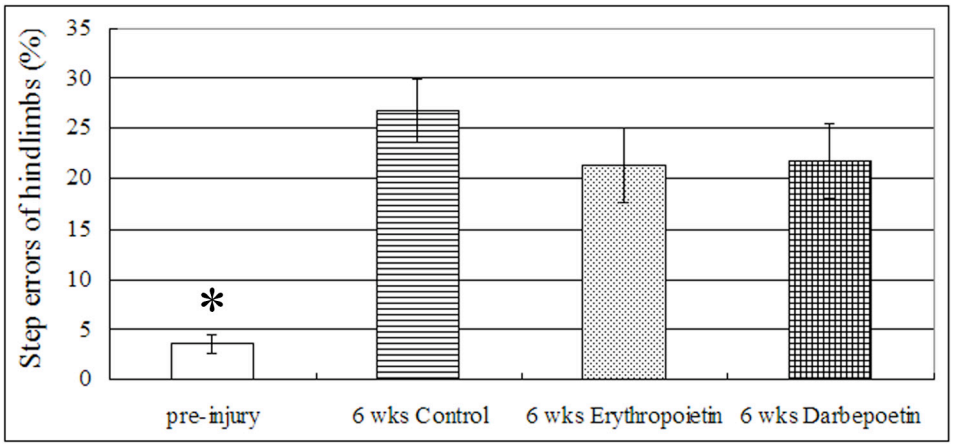


Figure 2.5.3: Footprint analysis

The base of hindlimb support is measured off of high-definition video recordings of the animal walking as the distance between the hindpaw placements. All animals demonstrate increased base of support at 6 weeks post-injury as compared to pre-injury (*, $p<0.01$). However, no significant improvements were noted in animals treated with erythropoietin or darbepoetin as compared to saline controls.

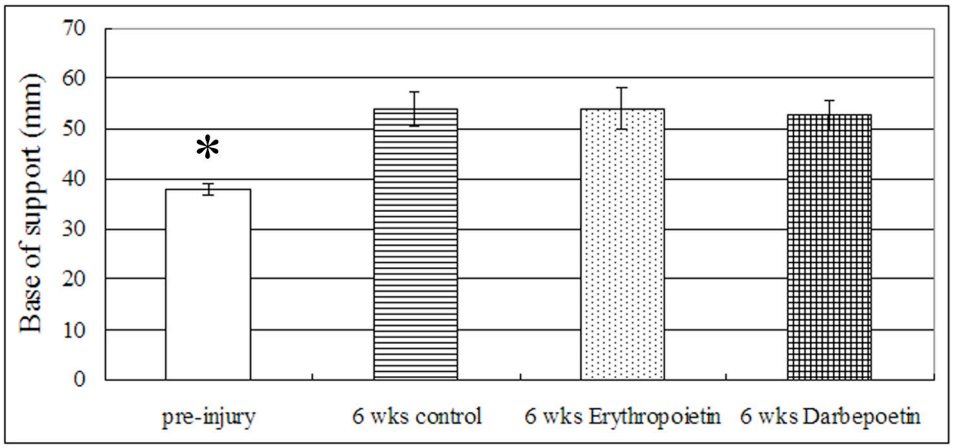


Figure 2.5.4: Sensory testing with von Frey Hair monofilament stimulation of hindpaw

At 6 weeks post-injury, the mechanical force threshold at which hindpaw withdrawal occurred increased significantly in all animals (*, $p<0.05$), suggesting a decrease in sensation. Neither erythropoietin or darbepoetin treatment altered this force threshold as compared to saline controls.

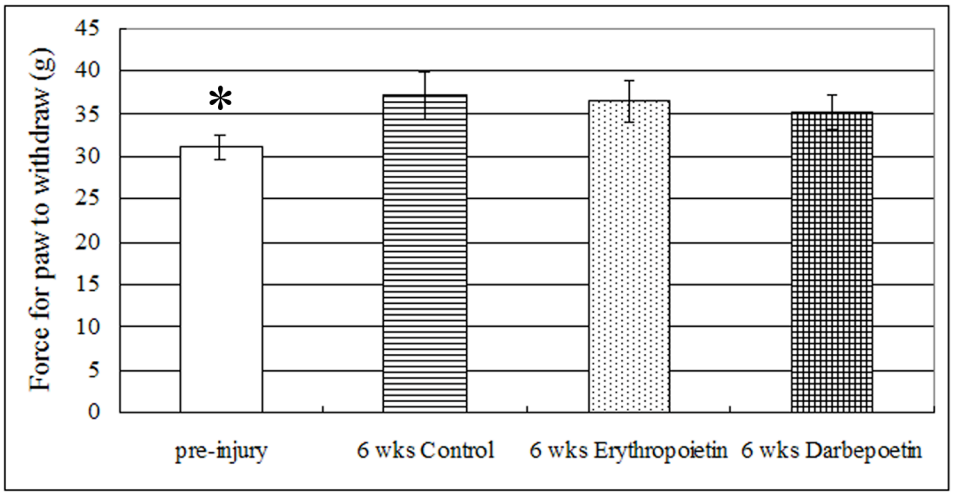
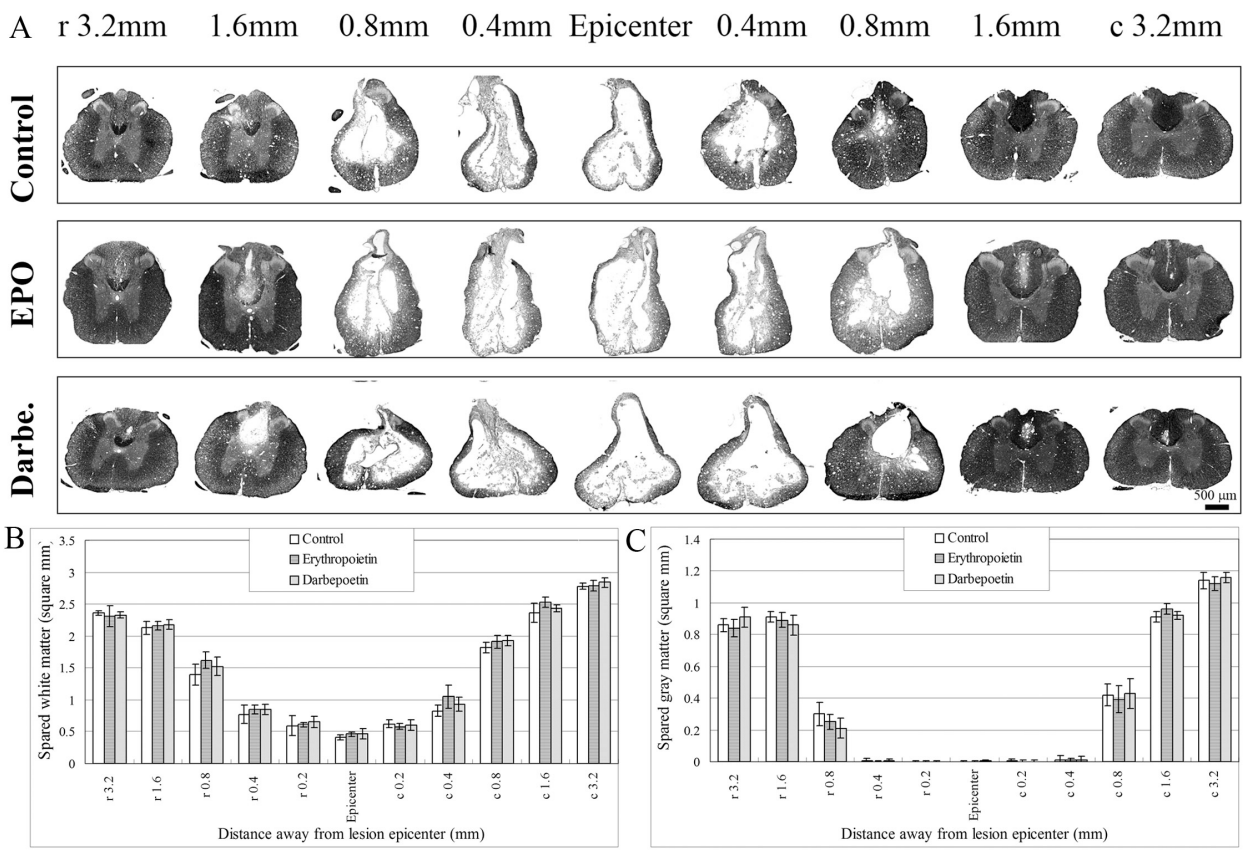


Figure 2.5.5: Histologic analysis of white and grey matter sparing at and around injury epicenter.

A, representative microphotographs of cross spinal sections six weeks after SCI. B and C, quantification of white and grey matter sparing at the injury epicenter and the surrounding spinal cord revealed no neuroprotective effect with either erythropoietin or darbepoetin as compared to saline controls.



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CHAPTER 3

The comparison of orally administered atorvastatin and simvastatin in their ability to improve outcomes after spinal cord injury[3]

[3] A version of this chapter will be submitted for publication. Cody Mann, Jae H.T. Lee, Jie Liu, Anthea M.T. Stammers, Wolfram Tetzlaff, Brian K. Kwon (2008-2009). The comparison of orally administered atorvastatin and simvastatin in their ability to improve outcomes after spinal cord injury.

3.1 INTRODUCTION

Statins are well known and commonly prescribed by physicians for their cholesterol lowering abilities. Their mechanism of action is mediated by the inhibition of hydroxymethylglutaryl-coenzyme-A (HMG-CoA) reductase, an enzyme responsible for the rate-limiting step of cholesterol synthesis. Aside from their influences on cholesterol synthesis however, this class of drugs has recently been observed to modulate several other physiological processes. These "pleiotropic" effects are thought to exist because the inhibition of HMG-CoA reductase also halts the production of several lipid intermediates (to cholesterol synthesis) such as isoprenoids[1]. Isoprenoids happen to be important modulators of several intracellular signaling molecules and their depletion can influence many cellular events[1]. Interestingly, several of the pleiotropic properties of statins can be exploited in the setting of central nervous system (CNS) injury. Experimental studies in stroke, traumatic brain injury (TBI), and spinal cord injury (SCI) have demonstrated that statins can positively influence several facets of CNS pathology[2] (Table 1.5.3). For instance they possess antioxidant properties and have been observed to up regulate endothelial nitric oxide synthase (eNOS). Furthermore, statins are thought to have powerful immunomodulatory abilities[3]; they have been shown to inhibit the expression of several pro-inflammatory cytokines, which play important roles in mediating secondary damage after SCI[4-6].

We sought to evaluate two statins, atorvastatin and simvastatin, based on data previously obtained in other labs. Pannu et al[7] have previously reported that a 5mg/kg daily dose of atorvastatin, starting 2 hours after experimental SCI, reduces inflammation and neuronal cell death. More importantly, they also reported an extensive improvement in locomotor recovery after treatment. Simvastatin, to our knowledge, has not been evaluated for SCI. However, in an experimental model of stroke, simvastatin (20mg/kg) was neuroprotective when administered 3 to 6 hours after a cerebral ischemia[8,9].

Although both atorvastatin and simvastatin target the same enzyme, they differ in their clinical pharmacology. Simvastatin is more lipophilic, therefore having a greater propensity to cross the blood brain barrier and, hence, a greater potential to target injured CNS tissue[10]. This subtle but important pharmacokinetic difference could provide simvastatin with an increased biological effect in the setting of a CNS injury. Atorvastatin and simvastatin have previously been compared in two separate studies; however, these were in an experimental model of traumatic brain injury. Wang et al. showed no difference in the neuroprotection afforded by both drugs, however Lu et al. observed simvastatin to be superior at improving neurologic

recovery[11,12]. Because either drug could be utilized in a clinical SCI setting, strong rationale exists for at least attempting to identify which of the two is more neuroprotective in a preclinical SCI model.

I hypothesize that simvastatin will improve neurologic and histologic outcomes with greater efficacy than atorvastatin in a clinically relevant experimental model of spinal cord injury. Animals were treated with either atorvastatin or simvastatin at 5mg/kg/day and 20 mg/kg/day respectively - doses that have previously been demonstrated as neurprotective[7-9]. Treatment was started 1 hour post injury and continued for one week. Animals were monitored for 6 weeks and both sensory and locomotor recovery were closely observed. Furthermore, spinal cords were analyzed histologically; this included quantification of spared white / grey matter and immuno-histochemical analysis using markers for inflammation, glia, and axons.

3.2 MATERIALS AND METHODS

Animal Model and Surgical Procedures

18 male Sprague-Dawley rats weighing 320-340 gm were anesthetized with an intraperitoneal 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). A dorsal midline incision was made over the mid-thoracic spine to expose the posterior spinal elements at T9-10. A laminectomy was performed, and the bases of the adjacent spinous processes were secured with modified Allis clamps which then held the animal secure within a custom frame. With half of the animal's weight suspended, the animal was positioned under the Ohio State University (OSU) Impactor, and the impactor tip was gently lowered to apply a preload force of 0.2 kdyne onto the dura. The impactor was then triggered to deliver a 1.5 mm displacement injury at 300 mm/s. Animals were excluded if the peak force fell outside the range of 200-260 kdynes. The dorsal wound was then closed. (For detailed methodology on injury model and surgery, see appendix 6.1.1)

One hour later, animals were awake from anesthesia and were randomized to receive treatment. Drugs were obtained from a local pharmacy, crushed, suspended in saline, and delivered by oral gavage. Animals received atorvastain (Lipitor®, Pfizer Inc., New York, NY) at 5 mg/kg (n=4), simvastatin (Zocor ®, Merck & Co., Inc., Whitehouse Station, NJ) at 20 mg/kg (n=5), or saline (n=9). Treatment continued, once a day, for one week. Animal temperature was monitored and maintained at 37° Celsius in an incubator during the acute postoperative period to

avoid the potential neuroprotection afforded by hypothermia. Manual bladder expression was performed 3 times per day until reflexive voiding returned. The animals received a subcutaneous injection of buprenorphrine (0.02 mg/kg, Temgesic®, Reckitt Benkiser HealthcareLtd., UK) just prior to their surgery and then again on post-operative days 1 and 2. All animals' surgeries and care were conducted in accordance to UBC Animal Care guidelines.

Behavioral Outcome Assessment

For all behavioral outcome assessments, baseline scores were first established by evaluating the animals prior to being injured. All evaluations post-injury were performed by individuals blinded to treatment group.

Open-field locomotor testing was performed by two blinded examiners on days 2, 7, 14, 28, 35, and 42 days post-injury using the Basso, Bresnahan, and Beattie (BBB) locomotor scale and subscore[13,14]. All open-field locomotor testing was performed under the same conditions and at the same time period on each scheduled testing date. (For detailed methodology on behavioral assessment, see appendix 6.1.2)

At 42 days post-injury, the animals were videotaped as they walked over a horizontally laid ladder with unevenly spaced rungs. Subsequent analysis determined the number of times the hindlimb slipped between the rungs, this was counted as a ("footfalls"). As a final measurement of functional outcome, the number of footfalls was divided by the total number of steps taken to produce a "percentage of error" value.

The animals underwent hindpaw pinprick sensory threshold testing at 42 days post-injury using a von Frey monofilament device (Semmes-Weinstein monofilament, Stoelting Co., Wood Dale, IL) to evaluate whether mechanical allodynia was present. In brief, the animal was placed on an elevated grid and observed for 5 to 10 minutes to ensure that it was calm. When both hindpaws were evenly resting upon the rungs of the grid, the monofilament was pressed into the center of the volar surface of the hindpaw. The mechanical force at which hindlimb withdrawal occurred and the time from stimulus application to hindlimb withdrawal were recorded.

Anatomic Outcome Assessment

At 45 days post-injury, all animals were sacrificed with a lethal injection of Pentobarbital Sodium (107 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and then perfused with phosphate buffered saline followed by fixation with cooled 4% paraformaldehyde. A 15 mm segment of spinal cord centered on the injury site was harvested, post-fixed overnight

in 4% paraformaldehyde, cryoprotected in increasing doses of sucrose, and then snap frozen over dry ice. The cords were sectioned axially at 20 μm thickness for histological analysis of the injury site. The ability of the experimental treatments to preserve or "protect" grey and white matter of the spinal cord was evaluated. Spinal cord sections 200 μm apart were stained with Eriochrome Cyanine (EC) as described by Rabchevsky et al[15], counter-stained with Neutral Red, then imaged on a Zeiss Axioskop microscope at 5x objective. Regions of spared white and grey matter were manually traced and then quantified using SigmaScan Pro version 5.0.0 (Systat Software Inc.).

Immunohistochemistry was performed on spinal cord sections spanning the injury site. Various cellular markers were used to evaluate several parameters of injury and secondary pathology. The following antibodies were used: monoclonal rabbit anti-neurofiliment (NF, 1:500, Serotec), monoclonal mouse anti-myelin basic protein (MBP, 1:200, Chemicon), monoclonal mouse anti-APC (CC1, 1:100, Calbiochem), monoclonal rabbit anti-activated caspase3 (1:500, BD Pharmingen), and monoclonal mouse anti-glial fibrillary acidic protein (GFAP, 1:500, Sigma). (For detailed methodology on histologic assessment, see appendix 6.1.3)

Images were taken with a Zeiss Axioskop microscope at 5x objective. ImageJ software (NIH) was then used to analyze the images. In the lateral, regions of spared white matter, the number of oligodendrocytes, apoptotic oligodendrocytes, and spared axons were estimated by counting CC1+/hoecsht+ cells, CC1+/activated Caspase3+ cells, and NF+/MBP+ axonal profiles respectively. Astroglial scarring and the inflammatory response were estimated by determining the proportion of GFAP+ and ED1+ area on selected spinal cord sections, a method that has been described by Popovich et al[16]. Consistent thresholding was applied to the cross sectional images in order to remove non-specific background signal and enhance the quantification of GFAP+ or ED1+ labeling[16]. The GFAP+ area was determined in a sample area on the boarder lining the lesion cavity on the ventral aspect of the spinal cord and divided by the total area sampled to produce the "proportion" of GFAP+ area. On the other hand, ED1+ area was determined on the whole spinal cord cross section and divided by the total area of the section to produce the "proportion" of ED1+ area.

Statistical Analysis

All statistical analyses were performed using SPSS 10.0 for Windows. Differences among the treatment groups were tested using either a Student's t-test or a one-way analysis of

variance (ANOVA) with a least significant difference (LSD) multiple comparisons test when warranted. Differences with a P value less than 0.05 were considered statistically significant.

3.3 RESULTS

Behavioral testing on our animals revealed no evidence of mechanical allodynia. Using the von Frey Hair monofilament device to stimulate the hindpaw of injured animals, we were unable to see any signs of increased sensitivity and any differences amongst treated groups. The average force required to stimulate a paw withdrawal for atorvastatin and simvastatin treated animals (Figure 3.5.1), 32.19±2.46(g) and 38.63±1.93(g) respectively, was not statistically different than the average for saline treated animals, 37.12±2.75(g). Furthermore, saline and simvastatin treated animals required a larger force for withdrawal than the pre-injury average of 32.88±1.05(g), indicating a loss in sensation after injury.

Locomotor recovery was assessed through two behavioral tests, the BBB open field locomotor test and the horizontal ladder test to measure footfalls. With these we were able to evaluate recovery in gross and fine coordinated locomotion. Compared to saline controls, only simvastatin was able to increase recovery in the BBB test. This began at day 28 post injury continued on to day 45, the last day of testing (Figure 3.5.2). At this very last time point, simvastatin treated animals reached an average value of 13.2±1.02, which was significantly more than the saline control value of 11.3±0.23 (Figure 3.5.2 A). These results were reflected in the BBB subscore analysis (Figure 3.5.2 B), as simvastatin significantly improved subscores at day 28 and day 45 post injury. On the last day of testing, simvastatin treated animals improved to an average BBB subscore of 9.2±1.07 and saline treated animals remained at 4.44±1.39.

Interestingly, locomotor improvement was not observed in the footfall analysis, as assessed by the percentage of hindlimb step errors while walking across a horizontal ladder. This error rate increased from a pre injury value of 2.67±0.63% to 30.38±4.08% for the saline control group (Figure 3.5.3). However, the error rates for neither simvastatin (28.37±3.18%) nor atorvastatin (27.63±7.30%) treated animals were significantly different than that observed in the controls.

Histological evaluation of the spinal cord 45 days after contusion injury showed the presence of a prominent cavity at the lesion center, surrounded by a rim of preserved tissue (Figure 3.5.4). Staining intact tissue with Eriochrome Cyanine allowed us to quantify white and

grey matter. In accordance with our behavioral results, this analysis revealed that simvastatin, and not atorvastatin, significantly spared white matter as compared to saline control. The average area of white matter at the injury epicenter in simvastatin, atorvastatin, and saline treated groups was 0.78±0.04mm^2, 0.50±0.18mm^2, and 0.41±0.04mm^2 respectively (Figure 3.5.4 B). To achieve a more representative estimate of total tissue sparing, the spared white matter area was summed over 0.4mm spanning across the injury epicenter. Doing so gave an average spared area of 2.40±0.11mm^2, 1.63±0.49mm^2, and 1.61±0.189mm^2 for simvastatin, atorvastatin, and saline groups respectively (Figure 3.5.4 C). Once again, statistically significant improvements in white matter sparing were observed with simvastatin treatment. Identical analyses of spinal cord grey matter revealed no significant sparing differences between treatment groups.

A more detailed analysis of the injury epicenter was performed using immunohistochemistry. With antibodies specific for axons (anti-neurofiliment, NF), myelin (anti-myelin basic protein, MBP), oligodendrocytes (CC1-antibody), and an apoptotic marker (anti-activated caspase-3) we were able to evaluate axonal and oligodendrocyte sparing. Myelinated axons that were NF and MBP positive were counted in spinal cord cross sections of the injury epicenter (Figure 3.5.5 A-C). The average number of myelinated axons counted in saline, atorvastatin, and simvastatin treated groups were 498±41, 590±42, and 808±122 respectively (Figure 3.5.5 D). In accordance with our white matter and behavioral analyses, simvastatin treated animals averaged a greater number of axons than animals treated with saline control. However, at a P value of 0.0601, these results failed to reach statistical significance (P value = 0.05). Similarly, a count of CC1 and hoecsht positive oligodendrocytes indicated an increase that was not statistically significant (Figure 3.5.6 A-D). Averaged counts of oligodendrocytes in saline, atorvastatin, and simvastatin treated groups were 33.3±5.8, 28.8±8.1, and 47.2±4.2 respectively. Comparing simvastatin to the saline control gave a non-significant P value of 0.0866. Lastly, a second count of oligodendrocytes that were also positive for activated caspase-3, an apoptotic marker, revealed that simvastatin treatment reduced the apoptosis of oligodendrocytes 45 days post injury (Figure 3.5.7 A-D). However, this too was non significant (P value = 0.21). Data for this analysis was presented as the proportion of apoptotic oligodendrocytes in the total number of oligodendrocytes counted. This gave values of 0.44±0.13, 0.31±0.062, and 0.25±0.051 for saline, atorvastatin, and simvastatin treated groups respectively.

Continuing on with further immunohistochemical analysis of the injured spinal cord, we observed that simvastatin reduces inflammation and increases astrogliosis. Using an antibody specific for activated microglia and blood borne macrophages (ED1 antibody), we were able to evaluate the extent of microglial activation and macrophage invasion 45 days after spinal cord injury (Figure 3.5.8 A). ED1 positive area was expressed as a proportion of the total cross sectional area of the cord. Average proportions for saline, atorvastatin, and simvastatin treated groups were 0.26±0.03, 0.23±0.02, and 0.17±0.01 respectively and only the simvastatin treated group was significantly less than saline control (Figure 3.5.8 B). The astroglial scar was quantified using a GFAP antibody (Figure 3.5.9 A). GFAP positive area was determined in a sample area that immediately surrounded the lesion cavity. This was expressed as a proportion of the total area sampled at the injury epicenter. Average values of 0.73±0.07, 0.72±0.09, and 0.55±0.07 were recorded for saline, atorvastatin, and simvastatin treated groups respectively and there were no significant differences between the groups (Figure 3.5.9 C). This process was then repeated in sections that were 0.6mm rostral and caudal to the injury epicenter. Rostral values were 0.34±0.01, 0.42±0.04, and 0.48±0.04 for saline, atorvastatin, and simvastatin treated groups respectively (Figure 3.5.9 B). Here, the value for the simvastatin treated group was significantly greater than control. The caudal values were 0.41±0.07, 0.46±0.13, and 0.54±0.07 for saline, atorvastatin, and simvastatin treated groups respectively and there were no significant differences between groups (Figure 3.5.9 D).

3.4 DISCUSSION

In this experiment, simvastatin was observed to improve functional recovery in a clinically relevant model of SCI. Although these improvements were not reflected in the footfall analysis, a test of fine locomotion, they were evident in the BBB locomotor test. This scale offers a well-established method of evaluating gross locomotor recovery but it fails to assess fine motor skills such as the ability to accurately place a paw on a thin metal wire. From our results, it seems that simvastatin, not atorvastatin, improves gross locomotor recovery.

Mechanical allodynia has been reported to occur after a contusion injury to the rat spinal cord, however our particular injury paradigm did not elicit this neurological phenomena[17,18]. The discrepancy between our results and those of others can be explained by differences in the severity and the anatomical location of SCI. We observed that most animals became less sensitive to mechanical stimuli at the hind paw, indicative of sensory paralysis, as apposed to mechanical allydynia which describes increased sensitivity. The lack of functional improvement after treatment could be in part due to a failure of the drugs to significantly protect enough sensory axons or neurons after this type of injury. Indeed, when the spinal cords were examined, it was evident that the dorsal columns were completely absent at the injury site. It is apparent that in our injury model the major ascending sensory systems are severely damaged. With the major sensory systems almost completely wiped out, there would be little room for a therapeutic agent such as simvastatin to improve sensory outcomes.

Analysis of spinal cord revealed that there was significantly more white matter and a greater number of myelinated axons at the injury site of simvastatin treated animals, but not atorvastatin treated animals. Furthermore, most of this sparing effect was observed in the lateral and ventral aspects of the spinal cord. Damage to supraspinal motor projections involved with fine motor control, such as the corticospinal tract, did not appear to be attenuated by simvastatin. These results are in agreement with neurologic outcomes and might explain why neurological recovery in fine motor skills was not influenced with simvastatin treatment but was improved in the gross locomotor ability of the same animals.

Oligodendrocyte survival was assessed at the injury center to explore other possible mechanisms for the improved locomotor recovery. Oligodendrocytes have been shown to undergo extensive apoptotic cellular death in the days/weeks following contusion to the spinal cord [19]. This in turn is believed to contribute to functional losses that can be mitigated by replenishing the oligodendrocyte population after SCI[20,21]. Interestingly, statins have been

reported to prevent oligodendrocyte death and increase oligodendrocyte survival and differentiation in experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis)[6]. This is further supported by in vitro studies showing that simvastatin increases oligodendrocyte differentiation and process extension[22]. Pannu et al[23] suggested that atorvastatin treatment, started prior to SCI, reduces oligodendrocyte apoptosis. However, they did not report these results in a similar study where atorvastatin treatment began after SCI[7]. We have shown that simvastatin delivered after SCI can attenuate oligodendrocyte loss. Although, these results failed to reach statistical significance, they could nonetheless play a part in the observed improvement in motor recovery of simvastatin treated animals.

Amongst the pathological milieu that occurs after SCI, inflammation is believed to play a major role in secondary damage to the cord[24]. The activation of microglia and the infiltration of peripheral macrophages are thought to contribute to inflammation as a whole. These cells facilitate inflammation and are believed to be responsible for at least some of the overall damage to the spinal cord[24]. In support of this are studies that have attenuated the macrophage response after SCI and have shown positive neurologic outcomes as a result[14,25,26]. Statins serve as powerful modulators of the immune system[3,27]. For example, Wang et al[12] observed that with statin treatment the inflammatory response after experimental traumatic brain injury can be attenuated. This included decreased microglial activation and was associated with an improved behavioral outcome[12]. In addition, Pannu et al[7,23] showed that atorvastatin treatment, starting before or after SCI, dramatically reduced microglial activation/macrophage infiltration. Although we did not observe such results with atorvastain, simvastatin treated animals did have a significantly reduced microglial/macrophage response. Thus, immuno-modulation by simvastatin may have mediated at least some of the positive results associated with this drug.

Intertwined with inflammation is the formation of a glial scar after SCI. Astrocytes respond to CNS injury by undergoing hypertrophy and producing numerous molecules that inhibit axonal growth. This response, referred to as reactive gliosis, isolates the injury site and contributes to the failure of axonal regeneration in the CNS[28]. On the other hand, the glial scar is also thought to have beneficial functions such as contributing to the repair of the blood brain barrier and limiting the migration of immune cells, inflammation, and subsequent secondary damage[28]. For example, experiments interfering with astrocyte migration and the formation of a glial scar have indicated that this process is indeed necessary to protect against tissue damage and necessary for normal functional recovery after SCI[29]. Recently, it has been reported that statins attenuate reactive astrogliosis after SCI[7]. This however does not agree with in vitro

studies that have suggested the opposite, where statins increase astrocyte activation[30,31]. In our hands, simvastatin significantly increased the astrogliotic scar lining the lesion cavity; this effect did not reach significance with atorvastatin. Our observations differ from those of Pannu et al[7], however it is possible that the effect of statins on astrogliosis differs temporally, since their analysis was done in the acute stage and ours was done 45 days post injury. The overall effect of increased gliosis on functional recovery remains difficult to interpret. Nevertheless, by forming a physical and chemical barrier for axonal regeneration, it is likely that increased gliosis is detrimental in a chronic setting[28].

Given that only simvastatin (20mg/kg) was efficacious at improving outcomes after SCI, it is evident that it is a better treatment than atorvastatin (5mg/kg) in the experimental paradigm used in this study. This indicates that the pharmacokinetic differences between the drugs may have an influence on the effect they have on CNS injury. There are inherent differences in bioavailability and lipophilicity between atorvastatin and simvastatin. For instance, because of differences in drug metabolism, atorvastatin has a much higher terminal half-life and is therefore more bioavailable [10,32]. Because of this, it remains difficult to compare the two drugs at the same dose. Nevertheless an attempt was made to correct for this difference in bioavailability as simvastatin was delivered at a higher dose. Furthermore, both these dosages have been reported as neuroprotective by other investigators[7-9]. Assuming that differences in bioavailability were corrected for, simvastatin had an apparent advantage in our experiments. This could possibly be explained by the fact that simvastatin is more lipophilic than atorvastatin and thus more readily able to cross the blood brain barrier [10,33]. This would subsequently give it a greater potential to attenuate secondary spinal cord pathology.

Others have shown that atorvastatin can potently reduce the secondary damage after SCI at the same dose[7]. Unfortunately, my experiments were unable to replicate these results. It is likely that this is due to differences in experimental procedure, as different injury paradigms and animal genders were utilized. For instance the gender of the animal can influence atorvastatins' bioavailability. We used male Sprague-Dawley rats in our experiments whereas Pannu et al used females of the same strain[7,23]. There are gender differences in the plasma concentration of atorvastatin after an oral dose in both rats and humans[34-36]. Therefore decreased bioavailability of atorvastatin in male rats may not have provided sufficient amounts of the drug to produce the same effect that was observed in females. In addition, there are reports indicating that the neuroprotective effect of atorvastatin, after experimental traumatic brain injury, is gender dependent[37]. More importantly though, we used a different injury model -the electromechanical

OSU impactor, as opposed to the weight drop device used by Pannu et al.[7,23]. The differences in injury biomechanics between these models could largely influence the pathological milieu that occurs after SCI and hence the action of a drug that is targeted against it[38,39]. Furthermore, Pannu et al. also injured their animals at the 12th thoracic spinal segment, where as we injured ours at the 9th/10th thoracic segments[7,23]. The different anatomical location of injury would change which neural pathways and internal spinal circuits (eg. central pattern generators) are injured and hence would also change the neurologic recovery that occurs afterwards. To that end, it is entirely possible that our particular combination of animal gender and injury model was not able to produce the same conditions and therefore the same biological effect that has been shown by others.

In conclusion, this study has shown for the first time (to our knowledge) that simvastatin can promote functional recovery in a clinically relevant model of SCI. This is supported by histological analyses, as simvastatin has a protective effect on spinal cord tissue and influenced various pathological events that are characteristic to CNS injury. Further study in the dosing regimen of this drug may expose an even greater efficacy and potentially pave way for future clinical trials.

3.5 FIGURES

Figure 3.5.1: Sensory testing with von Frey Hair monofilament stimulation of hindpaw

At 6 weeks post-injury, the mechanical force threshold at which hindpaw withdrawal occurred was measured. There was no evident increase in sensitivity at this time point; instead, animals seemed to become less sensitive. Neither simvastatin nor atorvastatin treatment significantly altered this force threshold as compared to saline controls.

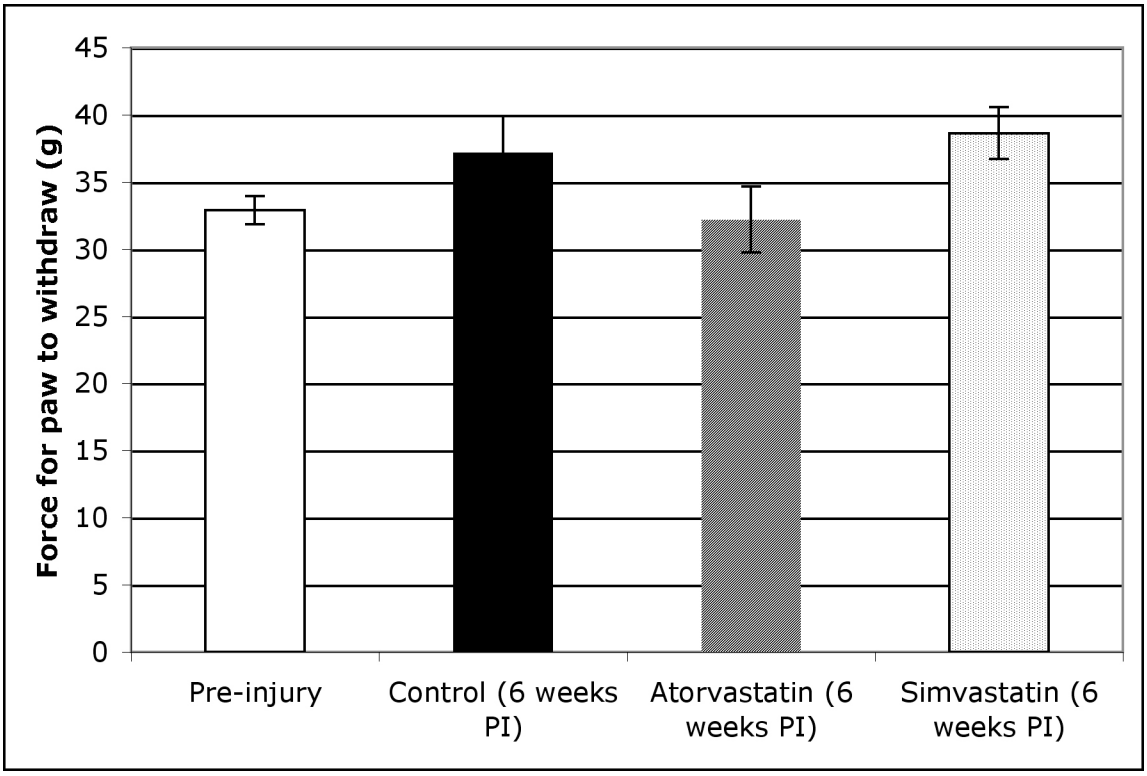


Figure 3.5.2: Open field locomotor function test (BBB scale)

Simvastatin significantly improved locomotor recovery starting 28 days post injury as compared to saline controls. Conversely, atorvastatin did not have a significant effect on locomotor recovery. These results were observed in both the BBB (A) and subscore (B) analyses.

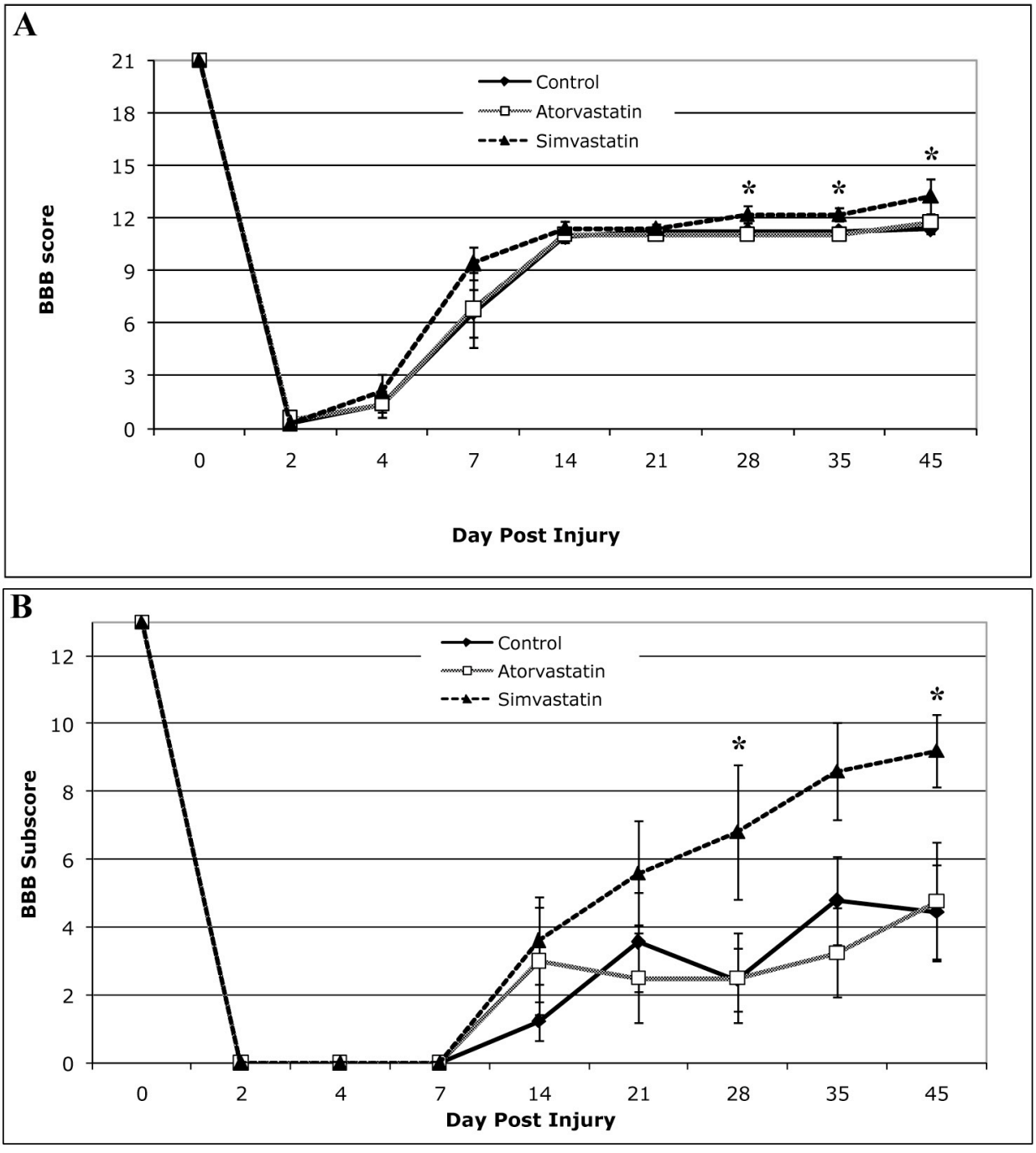


Figure 3.5.3: Horizontal ladder test to measure footfalls.

The number of footfalls (mistakes) that were made by an animal was divided by the total number of steps. All animals demonstrate increased footfalls 6 weeks post-injury as compared to pre-injury (*, $p<0.01$). No statistically significant improvements were observed in animals treated with atorvastatin or simvastatin as compared to saline controls.

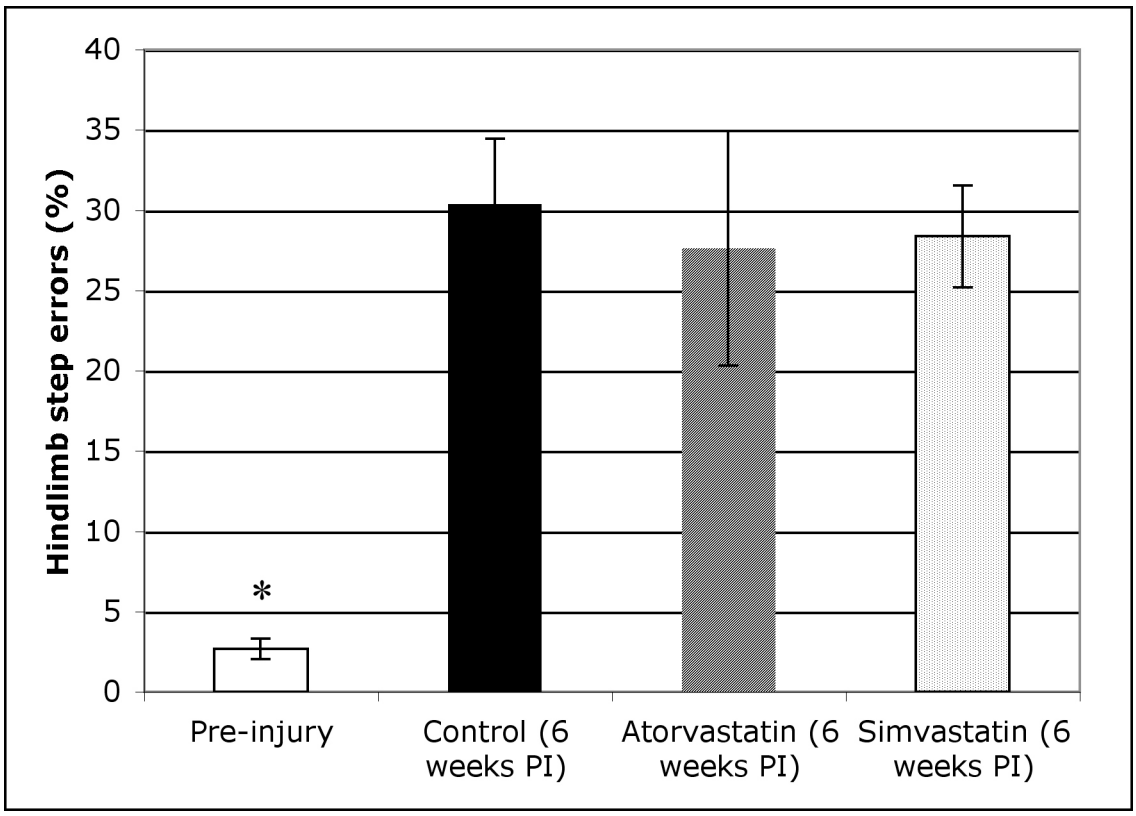


Figure 3.5.4: Histologic analysis of white and grey matter sparing at and around the injury epicenter.

(A) Representative microphotographs of spinal cord cross sections six weeks after SCI. (B) Quantification of white matter sparing revealed that simvastatin, not atorvastatin, had a significant (*, $p<0.05$) neuroprotective effect at the injury epicenter as compared to saline control. (C) Adding the spared white matter over a distance of 0.4mm surrounding the injury epicenter also showed that only simvastatin preserves more white matter than saline control (*, $p<0.05$). Similar analyses evaluating grey matter sparing, point-by-point (D) and spanning 0.4mm (E), revealed no neuroprotective effect with either atorvastatin or simvastatin as compared to saline control.

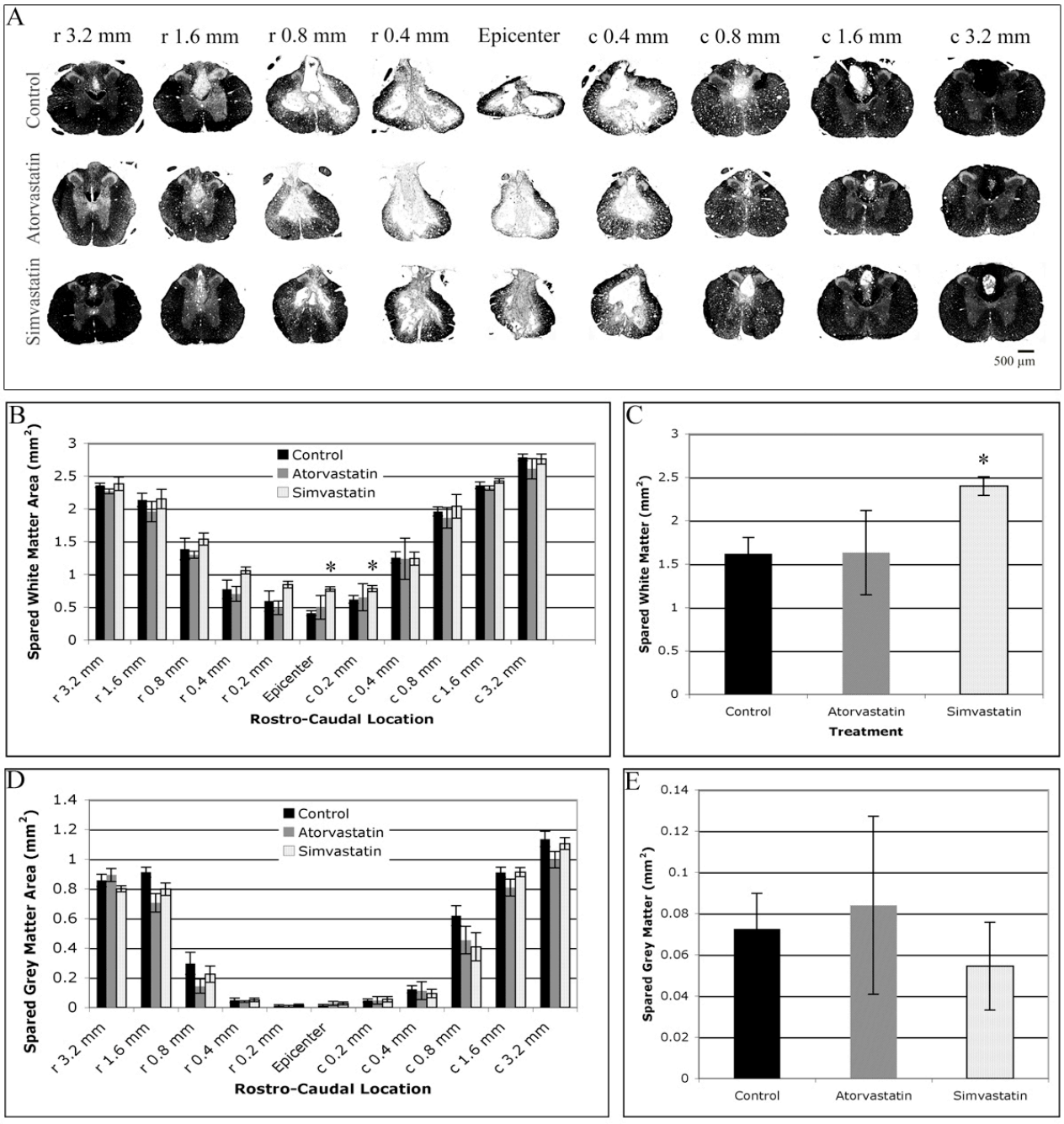


Figure 3.5.5: Histologic analysis of myelinated axons at the injury epicenter.

Spared axons (NF+, Red) at the injury epicenter that had an intact myelin sheath (MBP+, Green) were counted. Representative immunofluorescent images of saline control (A), atorvastatin (B), and simvastatin (C) treatment groups are presented. D, more axons were counted in animals treated with simvastatin (P=0.0601), however this value did not reach statistical significance.

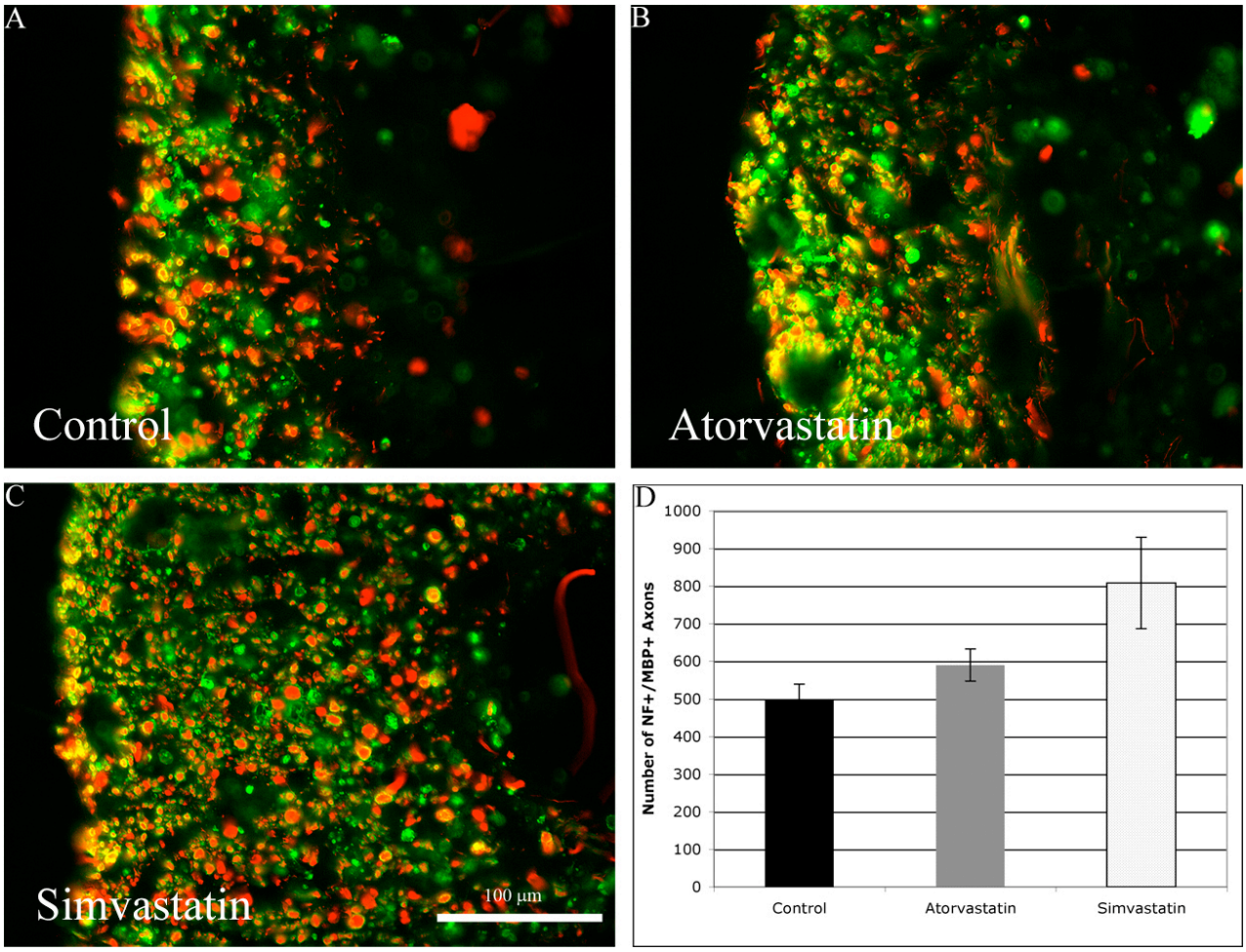


Figure 3.5.6: Histologic analysis of oligodendrocytes at the injury epicenter.

Oligodendrocytes (CC1+, green) with visible nuclei (hoechst+, blue) were counted at the injury epicenter. Representative immunofluorescent images of saline control (A), atorvastatin (B), and simvastatin (C) treatment groups are presented. D, a greater number of oligodendrocytes was present in animals treated with simvastatin (P=0.0866), however this value did not reach statistical significance.

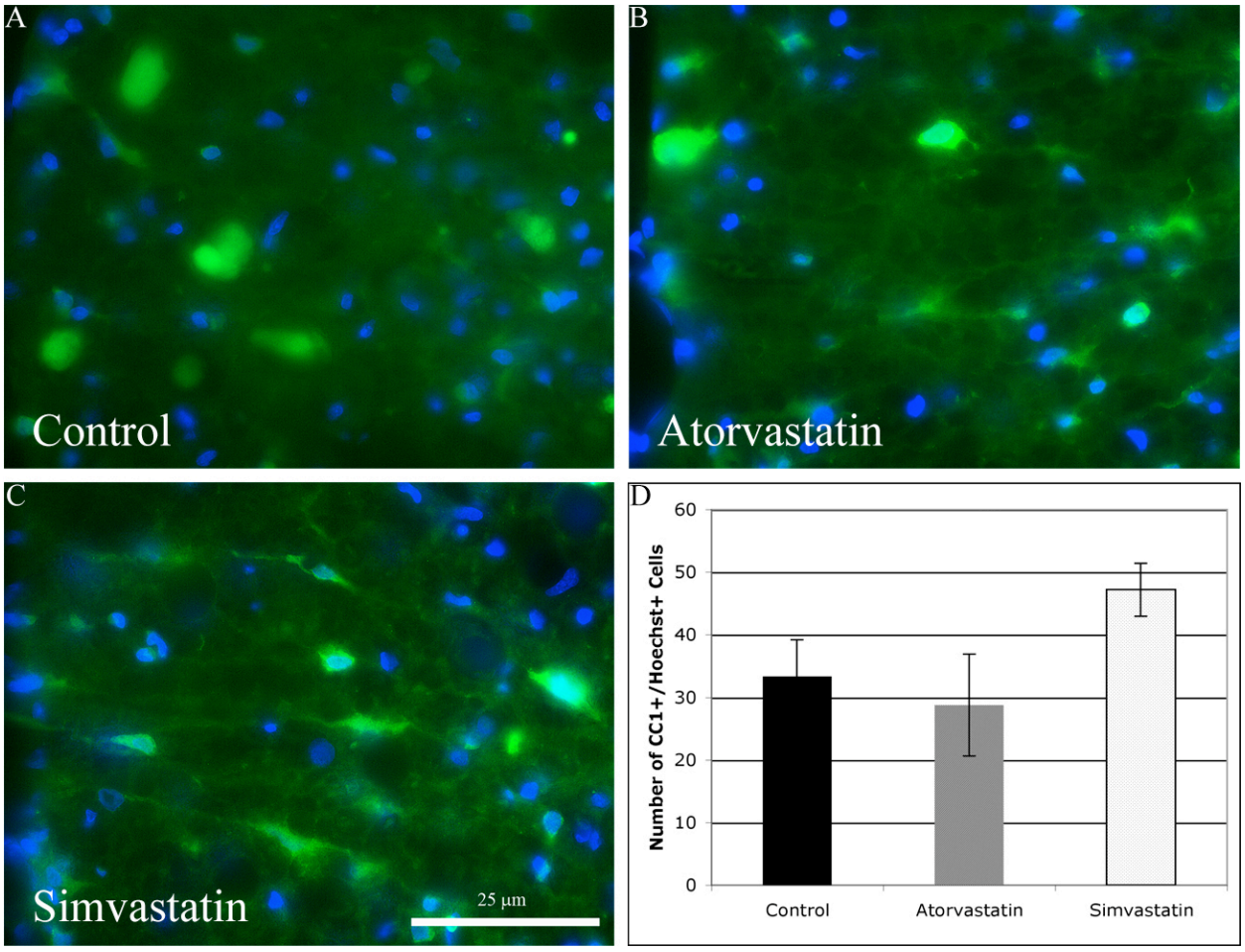


Figure 3.5.7: Histologic analysis of oligodendrocyte apoptosis at the injury epicenter.

Oligodendrocytes (CC1+, green) that were apoptotic (activated Caspase3+, red) were counted at the injury epicenter. Representative immunofluorescent images of saline control (A), atorvastatin (B), and simvastatin (C) treatment groups are presented. D, the amount apoptotic oligodendrocytes is expressed as a proportion of the total number of oligodendrocytes counted. Apoptosis was reduced in atorvastatin and simvastatin treatment groups, however these values did not reach statistical significance.

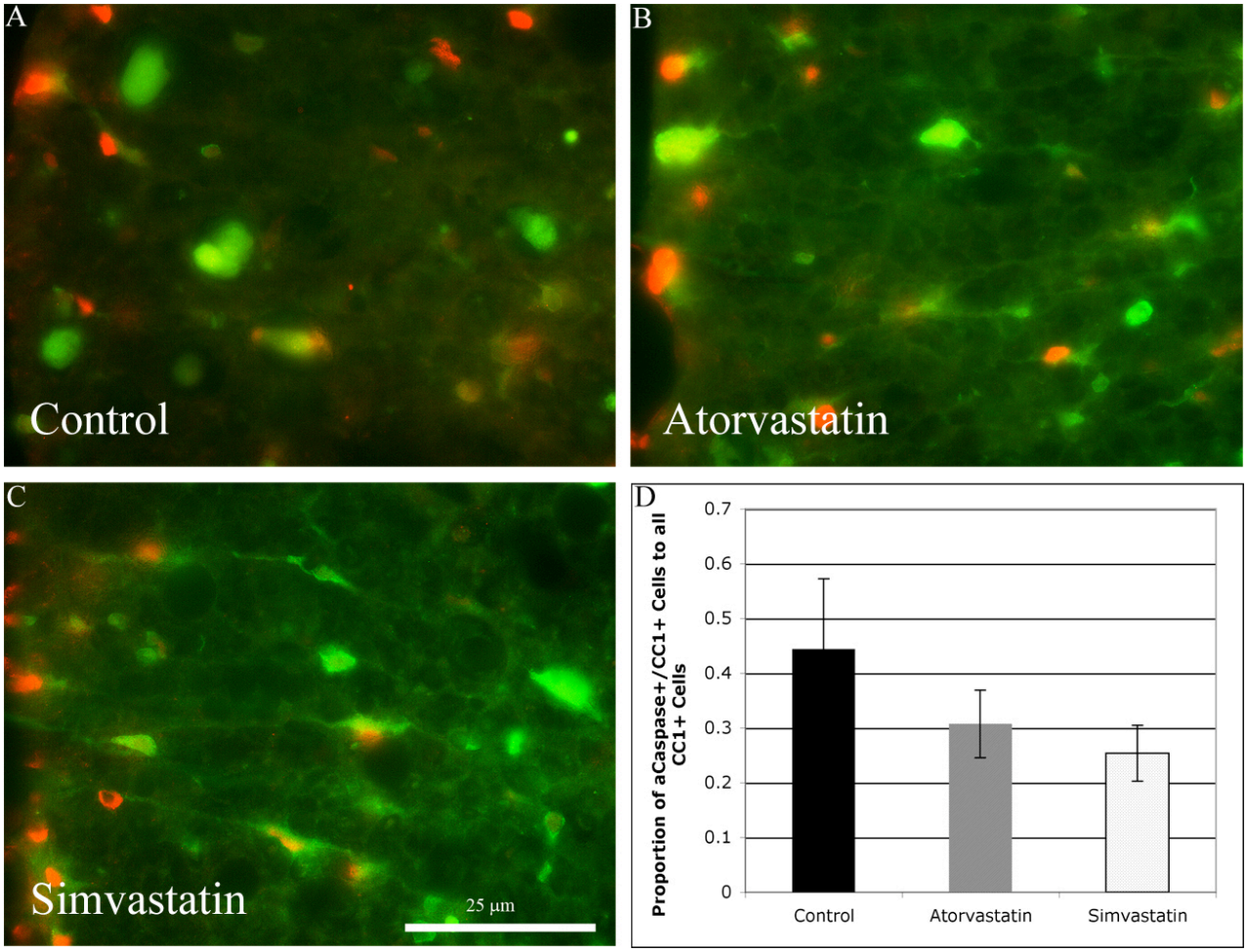


Figure 3.5.8: Histologic analysis of inflammation at the injury epicenter.

The activation of microglia and the presence of blood borne macrophages were quantified at the injury epicenter by immunostaining with an ED1 antibody. Representative images of saline control, atorvastatin, and simvastatin treatment groups are presented (A). Microglial activation or macrophage presence is expressed as the proportion of ED1 positive area in the total area of spinal cord cross section. (B). Simvastatin, not atorvastatin, significantly reduced (*, P>0.05) ED1 immuno-reactivity as compared to saline control.

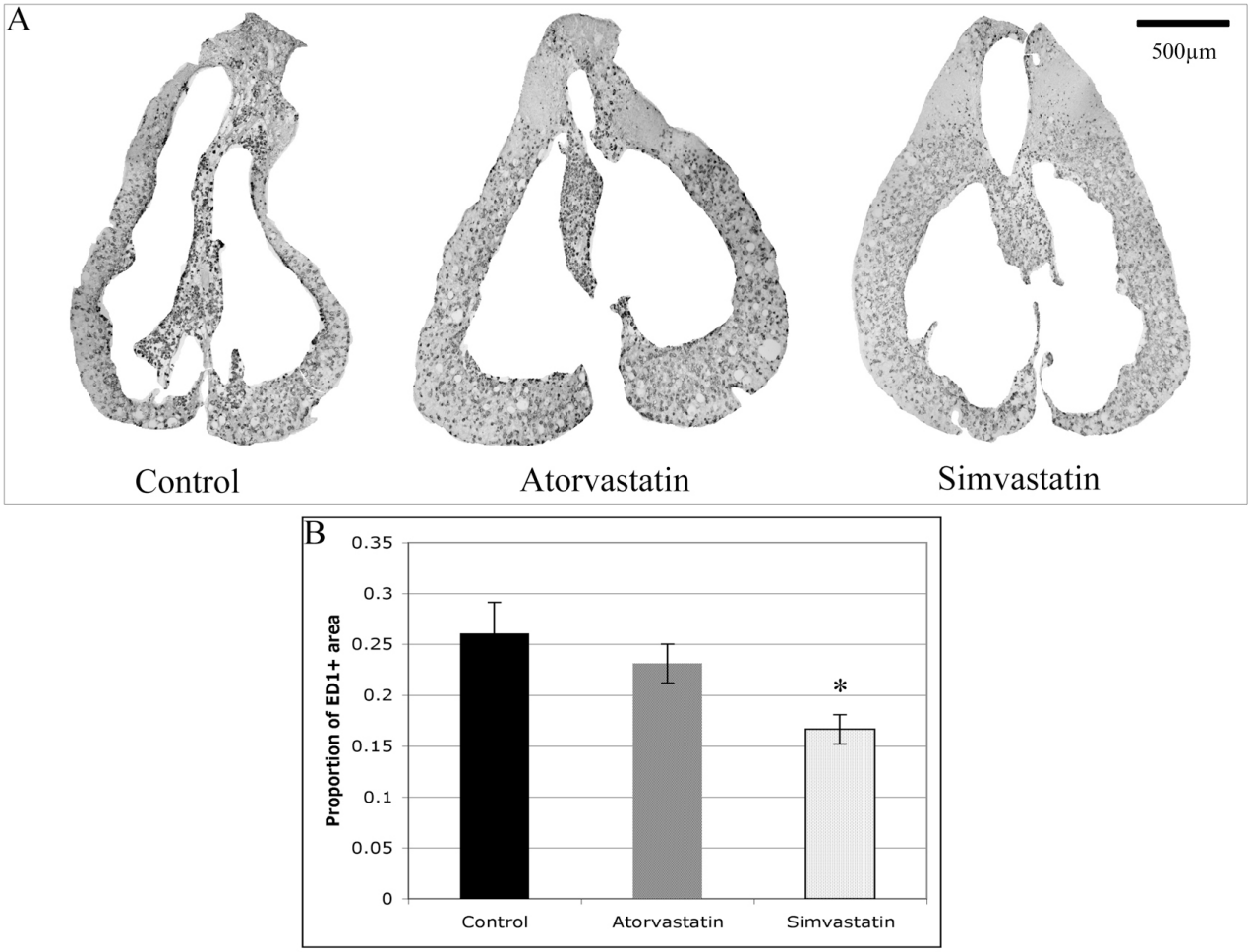
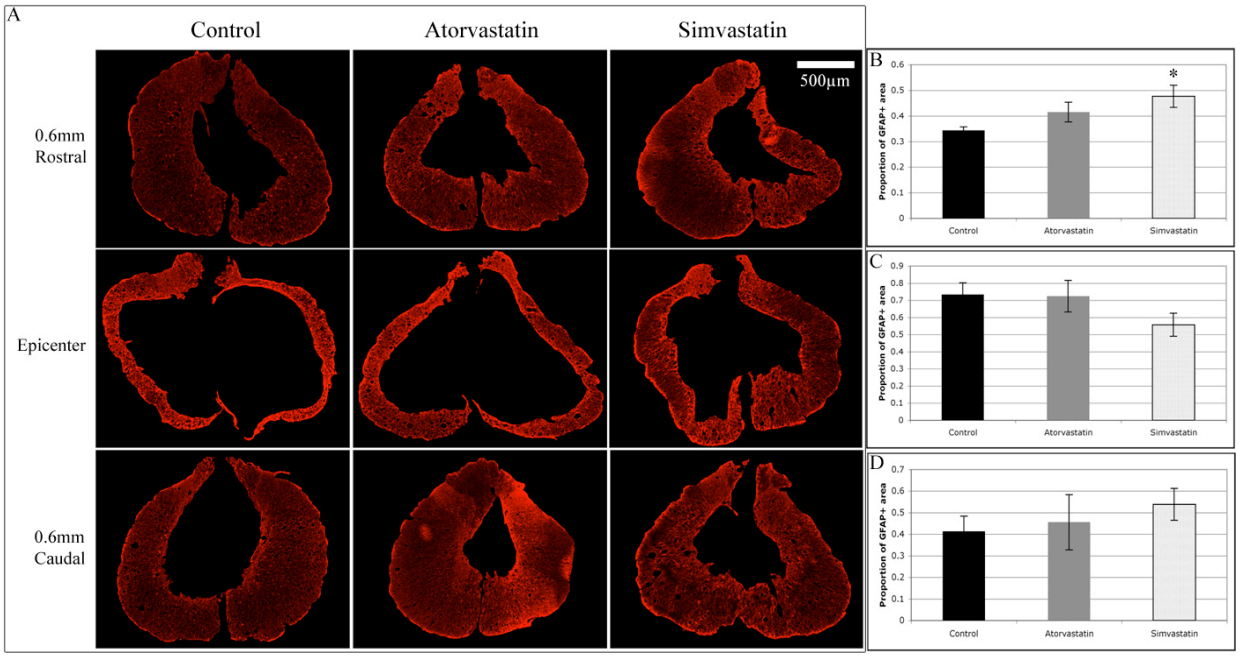


Figure 3.5.9: Histologic analysis of the astrogliosis at and around the injury epicenter.

The astrocytic scar was quantified by immunostaining for GFAP. Representative images of the injury epicenter and the tissue surrounding it are presented for each treatment group (A). Astrogliosis is expressed as the proportion of GFAP positive area in the total area of the spinal cord cross section (B, C, D). Simvastatin, not atorvastatin, increased GFAP immuno-reactivity rostral and caudal to the injury epicenter as compared to saline control. This effect was significant only at 0.6mm rostral (*, P>0.05) and was not present at the epicenter.



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CHAPTER 4

The effect of long-term subcutaneous simvastatin treatment on experimental spinal cord injury and its comparison with intraperitoneal minocycline treatment[4]

[4] A version of this chapter will be submitted for publication. Cody Mann, Jae H.T. Lee, Jie Liu, Anthea M.T. Stammers, Wolfram Tetzlaff, Brian K. Kwon (2008-2009). The effect of long-term subcutaneous simvastatin treatment on experimental spinal cord injury and its comparison with intraperitoneal minocycline treatment

4.1 INTRODUCTION

As described in the previous chapter, statins are a popular class of cholesterol lowering drugs that have shown biologic effects that extend far beyond their ability to inhibit cholesterol synthesis. These "pleiotropic" effects range from improving endothelial function and decreasing oxidative stress all the way to modulating the immune system[1]. In theory, all of these mechanisms could potentially inhibit secondary damage after central nervous system (CNS) injury. Our previous studies have already demonstrated that simvastatin has the ability to improve the neurologic recovery in a validated rat model of spinal cord injury. In this experiment, both behavioral and anatomical improvements were significant albeit modest, raising the question *"could the neuroprotective effects be improved upon with a better dosing and delivery method?"* Furthermore, we also wondered if simvastatin could be combined with another emerging neuroprotective treatment, minocycline, to utilize the beneficial effects of both drugs. If effective, the combinatory treatment of simvastatin and minocycline could quickly enter clinical trials since both drugs are commonly prescribed drugs and "safe to use".

In our previous study, rats treated with simvastatin, once a day for a week, began to show significantly improved motor function four to six weeks after injury. This, being a relatively "late effect", indicates that simvastatin may be inducing a plasticity mediated increase in locomotor recovery. Interestingly, simvastatin has recently been shown to promote neurite outgrowth in the presence of inhibitory molecules in vitro[2]. If this were to hold true in an *in vivo* setting, the increased neuroplasticity that would result could enhance behavioral recovery. I therefore reasoned that extending the period of drug administration for the duration of the whole experiment, six weeks, may further enhance neuroplasticity and locomotor recovery. A study by Pannu et al., who administered atorvastatin for 6 weeks, saw a dramatic improvement of locomotor function after SCI[3]. I have decided not to use atorvastatin in this study due to its lack of efficacy in our previous study. On the other hand it remains very possible that extended simvastatin treatment may improve the enhanced neurologic recovery that was previously observed with the drug.

In my previous experiments simvastatin was delivered by oral gavage. Although this procedure has been and continues to be extensively used in drug testing studies around the world, from a practical perspective, it is not a pleasant procedure to be performed on a rat that has recently been subjected to SCI. It was discovered that the animals tend to be very irritable after injury and their struggling makes it difficult to administer the drug. It was felt that to

continue this for 6 weeks would not only be excessively harsh on the animals, but that it could also affect their performance in behavioral studies over the course of the experiment. Therefore, an alternative method of drug administration was pursued. A literature search revealed several studies that have successfully delivered simvastatin subcutaneously to improve neurological outcome in experimental models of stroke and traumatic brain injury[4-10]. We therefore chose to proceed with a subcutaneous mode of administration, knowing from experience that giving a subcutaneous injection to a rat after SCI is much less problematic than oral gavage.

In the last decade, minocycline, a commonly prescribed antibiotic, has demonstrated a remarkable ability to alleviate numerous neurologic disorders. Experimental models of Amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, Huntington's disease, stroke, and SCI have all been successfully treated with minocycline[11]. At least four independent laboratories, including ourselves, have reported that minocycline treatment is neuroprotective following SCI in rodents[12-15]. Importantly, we have all shown that this drug is able to enhance locomotor recovery after SCI[13-15]. Although the mechanisms by which minocycline ameliorates central nervous system injuries remain incompletely understood, in the setting of SCI, it is believed that it can reduce neuronal cell death, inflammation, and axonal dieback[13-15].

The multifaceted cascade of secondary damage that is initiated by injury to the spinal cord is complex. Numerous pathophysiologic processes, including ischemia, inflammation, oxidative stress, electrolyte disturbances, and excitotoxicity contribute to neuronal damage and loss after SCI. Given the complexity of this pathophysiologic response, it would be rather unlikely for a single drug to attenuate all aspects of it. The corollary to that statement, therefore, is that a combination of agents would be more likely to attenuate more aspects of the secondary injury response than an individual drug alone. Given the efficacy of minocycline and simvasatin after acute neurotrauma and the mechanisms by which they act, there is reason to believe that combining these drugs may have an additive effect. Minocycline, for example, is thought to chiefly work by attenuating the activity of matrix metalloproteinases, inhibiting the activation of microglia, and inhibiting apoptotic mechanisms (reviewed by Zemke and Majid[16] and Stirling et al[17]). Statins on the other hand inhibit leukocyte infiltration and appear to augment a more "anti-inflammatory" Th2 cytokine response while suppressing the expression of the inflammatory Th1 cytokine response (reviewed by Arnaud et al[18]). While there may be some overlap in the pathways affected by these pharmacologic agents, the overlap is most likely incomplete, making it possible for the two agents to have an additive effect with combined treatment.

Therefore, **a hypothesis of the following study is that the combinatory treatment of minocycline and simvastatin will confer greater neuroprotection and locomotor recovery than either treatment alone in an experimental model of spinal cord injury**. Furthermore, **I also hypothesize that an extended regimen of simvastatin, for six weeks, will result in a substantially increased neurologic recovery after experimental SCI, as compared to the vehicle control**. Minocycline will be delivered via intraperitoneal injection, starting with a 90mg/kg dose one hour after injury and followed by a 45mg/kg dose every 12 hours for 3 days. This regimen has been efficacious in several studies utilizing a rat model of thoracic SCI[14,15,19]. Simvastatin, on the other hand, will be delivered subcutaneously at 20mg/kg/day starting one hour after injury, for three days, and 5 mg/kg/day thereafter. This, in turn is substantiated by studies with simvastatin that have demonstrated it to be efficacious at improving neurological outcomes in experimental models of stroke and traumatic brain injury when administered subcutaneously at dosages ranging from 0.2mg/kg to 20mg/kg[4-10] (Table 1.5.3).

4.2 MATERIALS AND METHODS

Animal Model and Surgical Procedures

62 male Sprague-Dawley rats weighing 290-320 gm were anesthetized with 2-4% isoflurane in oxygen and a dorsal midline incision was made over the mid-thoracic spine to expose the posterior spinal elements at T9-10. A laminectomy was performed, and the bases of the adjacent spinous processes were secured with modified Allis clamps that then held the animal secure within a custom frame. With half of the animal's weight suspended, the animal was positioned under the Ohio State University (OSU) Impactor, and the impactor tip was gently lowered to apply a preload force of 0.2 kdyne onto the dura. The impactor was then triggered to deliver a 1.5 mm displacement injury at 0.31 m/s. Animals were excluded if the peak force fell outside the range of 190-260 kdynes. The dorsal wound was then closed. (For detailed methodology on injury model and surgery, see appendix 6.1.1)

One hour later, animals were awake from anesthesia and were randomized to receive treatment one hour after injury. Animals were given minocycline (purchased from Sigma, St. Louis, MO) through intraperitoneal injection at 90 mg/kg (n=16). Purified Simvastatin (acquired from Merck through a MTA) was activated by alkaline hydrolysis (as described by Endres et al[4] and Kugi et al[20]) and delivered subcutaneously at 20 mg/kg (n=16). Minocycline treatment was continued afterwards, with the group receiving an additional 45mg/kg injection every 12 hours

for 3 days. Simvastatin was also continued, with the group receiving a 20mg/kg/day injection for 3 days. The dose was then lowered to 5mg/kg/day after the 3rd day post injury, this then continued for the duration of the experiment (42days). This decrease in the amount of simvastatin administered was due to the toxic effects that were observed when the drug was given for longer than 5 days at 20mg/kg/day (via subcutaneous injection). Dropping the dose to 5mg/kg spared the animals from the toxic effects, which included weight loss and a general lack of activity. Co-treated animals received both treatments (n=13) in the same manner as described above. To control for the effect of the vehicle solution and different modes of administration, the simvastatin animals received via IP injection the vehicle solution used to dissolve the minocycline, and conversely, the minocycline animals received via subcutaneous injection the vehicle for the simvastatin. Lastly, control animals received both, minocycline and simvastatin vehicles also in the same manner as described above (n=17).

Animal temperature was monitored and maintained at 37° Celsius in an incubator during the acute postoperative period to avoid the potential neuroprotection afforded by hypothermia. Manual bladder expression was performed 3 times per day until reflexive voiding returned. The animals received a subcutaneous injection of buprenorphrine (0.02 mg/kg, Temgesic®, Reckitt Benkiser HealthcareLtd., UK) just prior to their surgery and then again on post-operative days 1 and 2. All animals' surgeries and care were conducted in accordance to UBC Animal Care guidelines.

To evaluate how these drugs influence the inflammatory response in acute stages of injury, when inflammation is at its greatest[21], animals were subdivided into two subgroups (Table 4.5.1). 20 animals were sacrificed at 3 days post injury to create an acute subgroup (to analyze inflammation). The rest were kept for 42 days to create a chronic subgroup. The chronic group was used to evaluate behavioral recovery and histological outcomes in later stages of injury.

Behavioral Outcome Assessment

For all behavioral outcome assessments, the animals were preconditioned to testing procedures and baseline scores were established prior to the animals undergoing SCI. All evaluations post-injury were performed by individuals blinded to treatment group.

Open-field locomotor testing was performed by two blinded examiners on days 2, 7, 14, 28, 35, and 42 days post-injury using the Basso, Bresnahan, and Beattie (BBB) locomotor scale and subscore[22,23]. All open-field locomotor testing was performed under the same conditions and

at the same time period on each scheduled testing date. (For detailed methodology on behavioral assessment, see appendix 6.1.2)

At 42 days post-injury, the animals were videotaped as they walked over a horizontally laid ladder with unevenly spaced rungs. Subsequent analysis determined the number of times the hindlimb slipped between the rungs, this was counted as a ("footfalls"). As a final measurement of functional outcome, the number of footfalls was divided by the total number of steps taken to produce a "percentage of error" value.

Lastly, animals were tested with the CatWalk gait analysis system[24] using 3 outcome measures of specifically chosen gait parameters: regularity index, average hindpaw base of support, and average hind paw intensity. (For detailed methodology on behavioral assessment, see appendix 6.1.2)

Anatomic Outcome Assessment

At 3 or 45 days post-injury, animals were sacrificed with a lethal injection of Pentobarbital Sodium (107 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and then perfused with phosphate buffered saline followed by fixation with cooled 4% paraformaldehyde. A 15 mm segment of spinal cord centered on the injury site was harvested, post-fixed overnight in 4% paraformaldehyde, cryoprotected in increasing doses of sucrose, and then snap frozen over dry ice. The cords were sectioned axially at 20 μm thickness for histological analysis of the injury site.

The ability of the experimental treatments to preserve or "protect" grey and white matter of the spinal cord was evaluated. Spinal cord sections 200 μm apart were stained with Eriochrome Cyanine (EC) as per described by Rabchevsky et al[25], counter-stained with Neutral Red, and then imaged on a Zeiss Axioskop microscope at 5x objective. Regions of spared white and grey matter were manually traced and then quantified using SigmaScan Pro version 5.0.0 (Systat Software Inc.).

To visualize the inflammatory response, spinal cord sections spanning the injury site were immuno-labeled with an ED1 antibody. This allowed the visualization of peripheral macrophages and activated microglia at the injury site[21]. A DAB (3,3'-Diaminobenzidine) staining protocol was used to eliminate the miscalculation of auto-florescent background cells as immune cells, a problem that arises with florescent immuno-staining. (For detailed methodology on histologic assessment, see appendix 6.1.3)

Images were taken with a Zeiss Axioskop microscope at 5x objective. ImageJ software (NIH) was then used to analyze the images. The inflammatory response was measured by determining the relative proportion of the spinal cord cross section that was stained by ED1. This allows for the quantification of macrophages and activated microglia in the spinal cord (as described by Popovich et al[21]). However to prevent the miscalculation of non-specific background signal on the section, threasholding was applied to isolate ED1 labeling. Thereafter, the area labeled with ED1 on the spinal cord cross section was calculated and divided by the total cross sectional area to give a "proportion". 3 spinal cord cross sections (per animal), from the epicenter of injury, were quantified and averaged to obtain a more accurate sample from each animal. (For detailed methodology on histologic assessment, see appendix 6.1.3)

Statistical analysis

All statistical analyses were performed using SPSS 10.0 for Windows. Differences among the treatment groups were tested using either a Student's t-test or a one-way analysis of variance (ANOVA) with a least significant difference (LSD) multiple comparisons test when warranted. Differences with a P value less than 0.05 were considered statistically significant. Standard errors are indicated where means are reported, including graphs which have standard error bars.

4.3 RESULTS

Animals treated with both simvastatin and minocycline as a combinatory treatment, did not survive to the chronic stages of injury. These animals were euthanized within the first week after injury due to clinical signs of drug toxicity, such as decreases in physical activity, weight loss, and mortality.

No neurological improvements were observed in response to the individual treatments of either minocycline or simvastatin. Gross locomotor ability was scored throughout the duration of the experiment with the BBB scale and BBB subscore. At 42 days post injury, simvastatin, minocycline, and control groups had average BBB scores of 12.32±0.79, 12.41±0.70, and 12.04±0.53 respectively (Figure 4.5.1, A). At no time point during the post-operative period was there any statistically significant difference between the groups ($p > 0.05$, Two-Way ANOVA multiple comparison). Similarly, the BBB subcores revealed no significant differences between the groups (Figure 4.5.1, B). For gait testing on the horizontal ladder, all animals demonstrated

increased numbers of stepping errors post-injury, but there were no significant differences between groups at 42 days post-injury ($P > 0.05$, Student-t Test) (Figure 4.5.2). After injury, the percentage of hindlimb stepping errors was 25.6 ± 3.3, 32.6 ± 6.7, and 29.9 ± 5.9 for simvastatin, minocycline, and control groups respectively.

The three parameters chosen from the Catwalk analysis system also showed no significant neurological improvements as a result of treatment ($P > 0.05$, Student-t Test) (Figure 4.5.3). The regularity index, a relative measure of coordination, decreased after injury and at 42 days post-injury was 87.65± 2.01%, 92.01±0.96%, and 85.04±4.47% for control, simvastatin, and minocycline groups respectively (Figure 4.5.3, A). The hind paw base of support, which indicates gait stability, increased after injury and at 42 days post injury was 33.53±1.13mm, 30.42±1.79mm, 29.40±2.17mm for control, simvastatin, and minocycline groups respectively (Figure 4.5.3, B). Lastly, the hind paw intensity, which estimates how much pressure or weight is supported on the hind paws, decreased after injury and at 42 days post injury was 58.01±5.86, 58.88±3.70, and 63.33±6.51 for control, simvastatin, and minocycline groups respectively (Figure 4.5.3, C). Once again, none of these measurements demonstrated significant differences amongst groups ($P > 0.05$, Student-t Test).

Histological evaluation of the spinal cord 45 days after contusion injury showed the presence of a prominent cavity at the lesion center, surrounded by a rim of preserved tissue (Figure 4.5.4, A). Staining intact tissue with Eriochrome Cyanine allowed us to quantify white and grey matter. In accordance with our behavioral results, this analysis revealed that neither simvastatin nor minocycline significantly spared white matter as compared to the control treatment. The average white matter area at the injury epicenter in control, simvastatin, and minocycline groups was 0.36±0.05mm^2, 0.43±0.07mm^2, and 0.43±0.06mm^2 respectively (Figure 4.5.4, B). To achieve a more representative estimate of the total tissue sparing, the spared white matter area was summed over 1.6mm spanning the injury epicenter. Doing so gave an average spared area of 4.28±0.27mm^2, 4.74±0.40mm^2, and 5.06±0.46mm^2 for control, simvastatin, and minocycline groups respectively (Figure 4.5.4, C). Once again no statistically significant improvements in white matter sparing were observed with any treatment ($p > 0.05$, Two-Way ANOVA multiple comparison and Student-t Test). Identical analyses of grey matter also revealed no significant sparing differences between treatment groups (Figure 4.5.4, D-E).

The macrophage and microglial response is a significant portion of the inflammatory response that occurs after spinal cord injury. It was evaluated by quantifying the amount of peripheral macrophages that had infiltrated into the spinal cord and the amount of microglial

activation. Both macrophages and activated microglia have similar morphology and are labeled with the ED1 antibody (Figure 4.5.5). At the injury epicenter, the relative proportion of spinal cord cross sectional area that was occupied by these cells was determined. Three days post injury, average proportions were 0.0417±0.006, 0.0383±0.005, 0.023±0.004, 0.020±0.002 for control, minocycline, simvastatin, and simvastatin plus minocycline groups (Figure 4.5.5, B). There was a significant reduction in the amount of macrophages/microglial activation in the simvastatin and simvastatin plus minocycline groups compared to control ($P > 0.05$, Student-t Test). 42 days post injury, average proportions of ED1 stained tissue were 0.25±0.017, 0.228±0.018, 0.24±0.012 for control, minocycline, and simvastatin respectively (Figure 4.5.5, C). In this later point however, there were no significant differences between control and treated groups ($P > 0.05$, Student-t Test).

4.4 DISCUSSION

Many of the observations in this study were unexpected and somewhat disappointing, considering that we initiated it based on the results of our previous work which demonstrated a positive neuroprotective effect with simvastatin, and with minocycline[13]. Of the two hypotheses, we were only able to fully address one of them. Unfortunately the combinatorial neuroprotective approach of minocycline and simvastatin was not assessed due to the consistent morbidity and mortality when administered together. We were unable to evaluate neurological recovery and tissue protection in this group, as the animals did not survive to the chronic stages of injury (because of drug toxicity). As for the second hypothesis of this study, the chronic administration of simvastatin did not improve neurologic recovery. This was somewhat of a surprise since our earlier experiments had already established simvastatin as a beneficial treatment for spinal cord injury, if delivered in acutely (first week after injury).

Adverse side effects from high dose administration of simvastatin in rats have been reported[26]. After oral delivery of 150-200mg/kg Smith et al.[26] observed dose-related increases muscle degeneration, decreases in physical activity, weight loss, and mortality. The dosages used to produce these side effects are quite high, and therefore we did not anticipate them with a 20mg/kg subcutaneous regimen. In our previous experiment, oral delivery of simvastatin at 20mg/kg for one week was well tolerated. Furthermore, others have used (in other injury models) a 20mg/kg subcutaneous regimen for at least a week, without any problems noted[4-10]. So it was surprising to see toxicity after 4-5 days of subcutaneous delivery at 20mg/kg.

These results suggest that the bioavailability of simvastatin differs substantially between oral and subcutaneous routes of administration. To our knowledge there is no study that has directly compared the pharmokinetics of subcutaneous versus oral delivery for simvastatin. It has however been demonstrated that the absorption of oral drugs is decreased after spinal cord injury due to impaired gastric emptying and decreased gastro-intestinal blood flow [27]. Therefore, after oral delivery in our previous study, the animals may have been getting substantially less simvastatin systemically than in with the subcutaneous delivery. Furthermore, spinal cord injury can decrease liver blood flow and hence decrease the metabolism of high extraction drugs (dependent on extensive liver metabolism) such as statins[27,28]. This would inevitably increase bioavailability and explain why others (using different injury models) were able to deliver the same dosage without any side effects. Overall, it is likely that the combination of our injury model and subcutaneous delivery method resulted in dangerously high levels of simvastatin and caused toxicity. By lowering the dosage to 5mg/kg after 3 days at 20mg/kg we were able to avoid this. Nonetheless, the lower dosage did not promote neurologic recovery.

Going into this study we did not expect that there would be a detrimental drug interaction between minocycline and simvastatin[29]. Drug interactions with simvsatatin seem to occur when one agent interferes with hepatic clearance or metabolism of simvastatin[29-31]. Minocycline has been safely delivered in several experiments at the same dosages we used without any serious implications[14,15,19,32]. On the other hand, separate reports have indicated that minocycline can cause hepatic toxicity[33]. The possibility of hepatic toxicity when both drugs were onboard could have interfered with simvastatin metabolism. It can be reasoned that the combination of high dose minocycline and simvastatin treatment, and our injury model probably burdened the animals with dangerously high levels of simvastatin and liver failure. Perhaps starting with a lower simvastatin and minocycline dose may avoid toxicity; this however remains complicated since the neuroprotective effects of both drugs may be lost as the dosages are lowered.

Simvastatin at 20 mg/kg was found to significantly attenuate the microglial/macrophage inflammatory response 3 days after spinal cord injury. Statins have a powerful ability to modulate many different aspects of the immune system[1,34]. Our results can be explained by at least several mechanisms. Statins have an anti-proliferative effect on lymphocytes and can decrease the expression of pro-inflammatory cytokines such as TNF and Il-1beta[34]. Furthermore statins can directly reduce the binding capacity of integrin adhesion molecules that are used by leukocytes to migrate across vascular walls into injured tissue. For instance, statins can bind to the integrin lymphocyte function associated antigen 1 (LFA1) to prevent interaction with

intercellular adhesion molecule (ICAM1)[1,34]. These possible mechanisms could prevent macrophages from accumulating within the spinal cord after injury. In contrast to what was seen 3 days post injury, no difference in the extent of microglial/macrophage staining within the cord was seen between simvastatin and control animals at 42 days post-injury. This could be explained by a decrease in biologic effect with the decrease in dosage from 20mg/kg to 5mg/kg (at 3 days post injury). In addition, 42 days after injury the microglial/macrophage inflammatory response has already peaked and is diminished[21], and so a substantial effect might not be expected. Therefore it is likely that since there is less of an inflammatory response to influence at this time point, the drug that was present was unable to visibly alter it.

These results are notably different from our previous ones, where it was observed that, after one week of oral simvastatin treatment, the microglial/macrophage inflammatory response was attenuated at 42 days post injury. Here, simvastatin treatment seemed to have an effect on the immunologic response that lasted for the duration of the experiment. It is possible that the lessened microglial/macrophage response was due to neuroprotection, decreased tissue damage, and a decreased immune response that was afforded by the simvastatin treatment. Neuroprotection and decreased tissue damage were not observed with chronic simvastatin treatment in this study. It is likely that the one-week oral treatment, at 20mg/kg, significantly altered the normal pathological and immune response in such a way that the changes it caused were still visible 5 weeks later.

In this experiment, simvastatin reduced the microglial/macrophage inflammatory response 3 days after SCI; yet, it failed to protect the spinal cord from secondary damage and improve neurologic recovery. This was, again, at odds with our earlier experiment where one-week treatment (20mg/kg/day) resulted in increased recovery and improved histologic parameters. Nevertheless, it is hard to compare the two studies since we were forced to lower the dosage before the end of one week. By lowering the dosage from 20mg/kg to 5mg/kg after 3 days we were able to avoid toxicity. Doing so may not have provided enough simvastatin to the animals to elicit the same biologic effects observed in our first experiment. Alternatively, the long-term administration of simvastatin could have very well negated any beneficial effects produced by one-week treatment. The pathological processes after SCI are complex; inflammation has both beneficial and harmful roles in the final outcome of SCI. We are certain that the drug influences the immune system, as evidenced by its effects on microglial activation and macrophage infilteration. It could be that the long-term treatment regimen altered inflammatory processes in a way that may have been detrimental. For instance, statins are known

to influence T-lymphocyte activity, which are recruited into the spinal cord during the later stages of injury [21,34]. It is possible that chronic simvastatin treatment attenuated T-lymphocyte mediated repair processes to negate the beneficial effects seen after one-week of treatment.

Another unexpected and disappointing result was the inability of minocycline to improve any of the tested outcomes. We chose to use a dosing regimen that has worked for others, albeit in a different injury model[14,15,19]. The cited studies all used a thoracic weight drop injury model, which differs from ours with respect to the biomechanical injury parameters[35]. There are no reported studies that have evaluated minocycline using the OSU Impactor as the primary injury device (Table 1.5.4). We conclude that minocycline or the dosing regimen in which it was delivered was not efficacious in our injury model. A quicker and better delivery method and a longer treatment period may both be ways to improve the action of minocycline after an OSU Impactor injury. This is substantiated by recent results from our lab in which an intravenous approach was used to deliver minocycline after the same injury, resulting in modest improvements in neurologic function (Unpublished results)

Although we were unable to produce any positive results with either minocycline or simvastatin, this does not exclude them as potential therapies for SCI. Given the extensive amount of literature and evidence that supports each treatment, further work on these drugs is warranted - to develop and identify the optimal single or combined treatment paradigm. Furthermore, it has become clear to us that different injury parameters and the strain of rat used can dramatically influence the outcome of an experiment. Therefore it would be worthwhile to evaluate and compare treatments in multiple injury models to elucidate the reasons for these differences – and decide which experimental model most accurately simulates a human injury. Once again, this would allow the development of an optimal treatment paradigm, which can then be tested in human clinical trials.

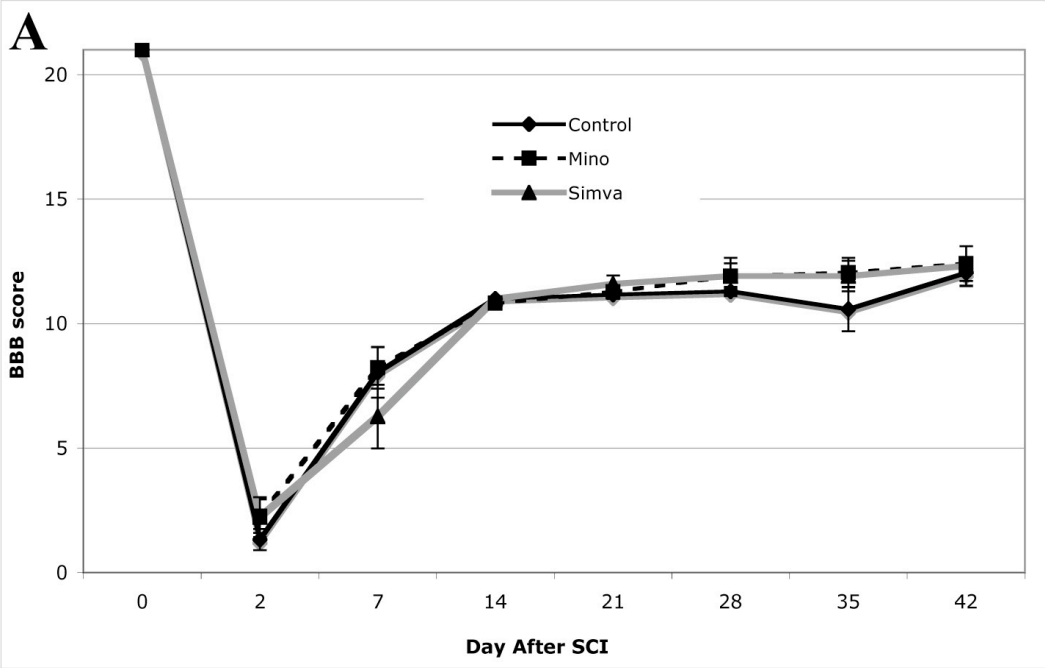
4.5 FIGURES AND TABLES

Table 4.5.1. Treatment groups.

	Simvastatin	Minocycline	Minocycline + Simvastatin	Control
Chronic Group (42 days)	n=11	n=11	n=8	n=12
Acute Group (3 days)	n=5	n=5	n=5	n=5
Treatment	Simvastatin 20mg/kg/day for 3 days (starting 1 hour after injury) + 5mg/kg for the remainder (39 days) + Minocycline vehicle.	Minocycline 90mg/kg 1 hour after injury + 45mg/kg every 12 hours for 3 days. + Simvastatin vehicle.	Both Simvastatin and Minocycline dosing regimens, as described.	Vehicle injection of both treatments in the same manner the actual drug was delivered to the other groups.

Figure 4.5.1: Open field locomotor function test (BBB scale)

None of the treatments what were tested appeared to improve gross locomotor recovery. There was no statistically significant ($p<0.05$) difference between the control, simvastatin, or minocycline treated groups in both the BBB (A) and subscore (B) analyses.



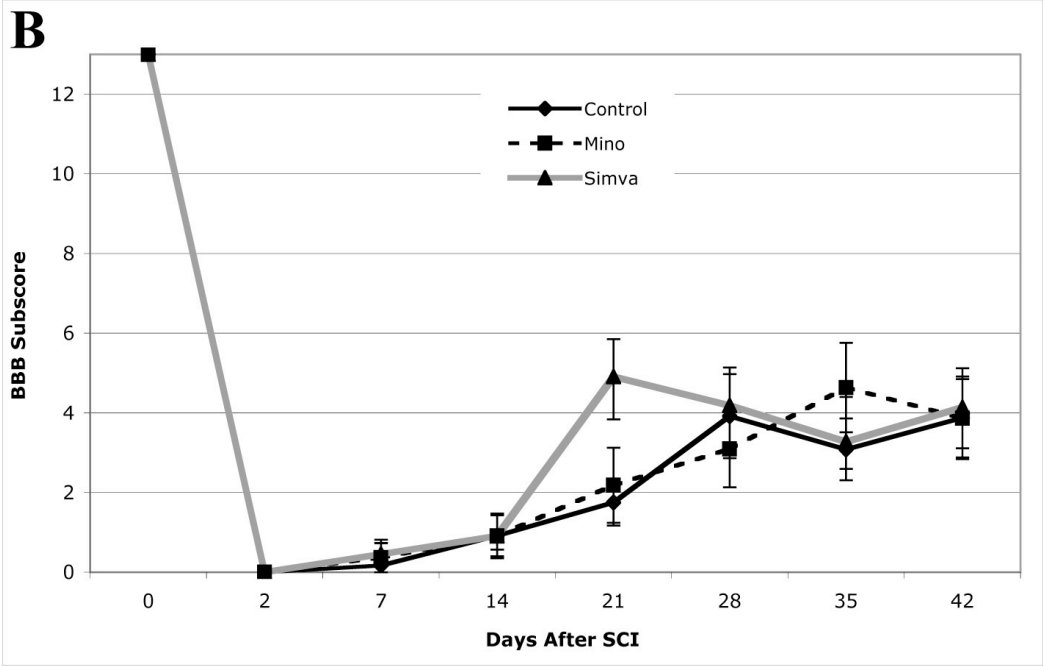


Figure 4.5.2: Horizontal ladder test to measure footfalls.

The number of footfalls (mistakes) that were made by an animal was divided by the total number of steps. All animals demonstrate increased footfalls 6 weeks post-injury as compared to pre-injury. However, no statistically significant ($p<0.05$) improvements were observed in animals treated with minocycline or simvastatin as compared to control animals.

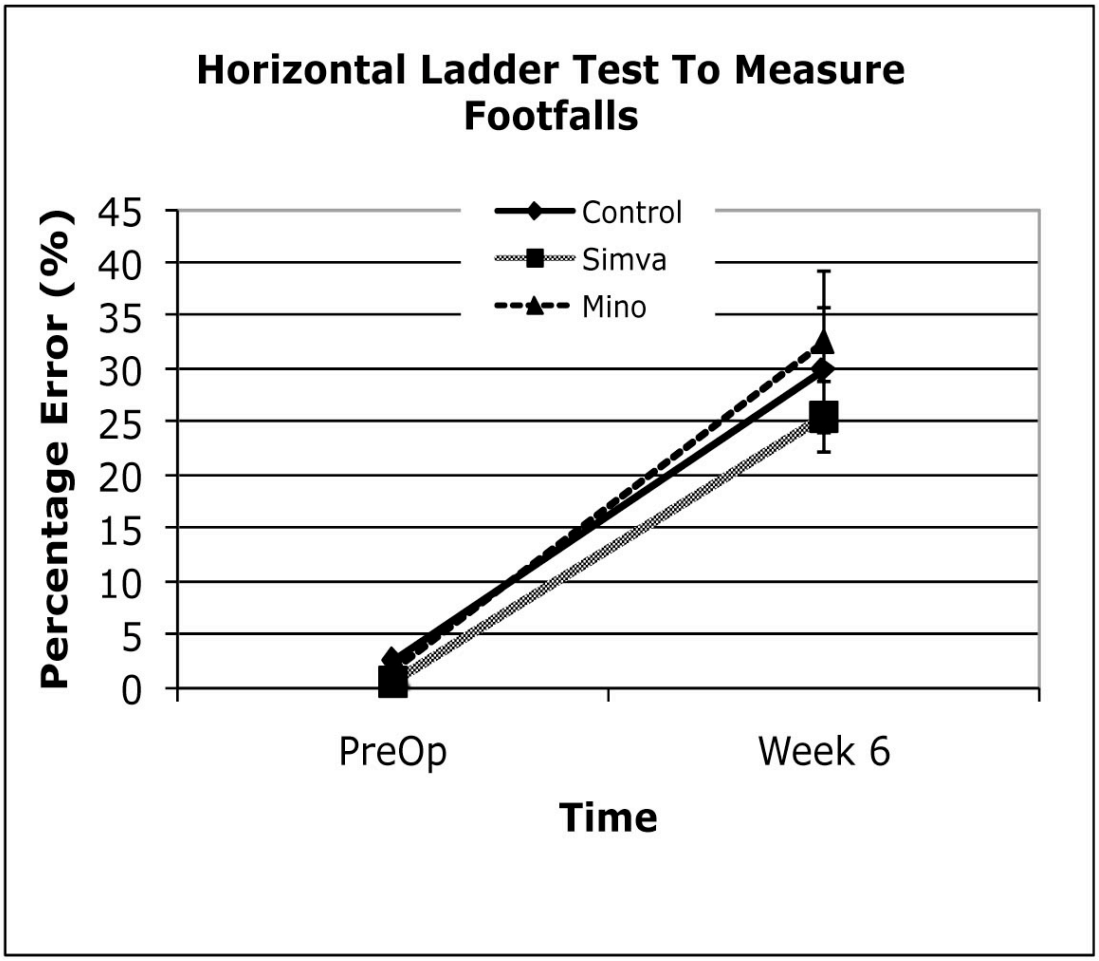


Figure 4.5.3: Catwalk gait analysis to evaluate motor recovery.

Three parameters were chosen to evaluate the recovery of locomotor function at 5 and 6 weeks post injury. The regularity index (A), hind paw base of support (B), and hind paw intensity (C) all demonstrated decreased motor function after injury followed by a period of recovery. However, neither minocycline nor simvastatin significantly ($p<0.05$) increased this recovery as compared to the control treatment.

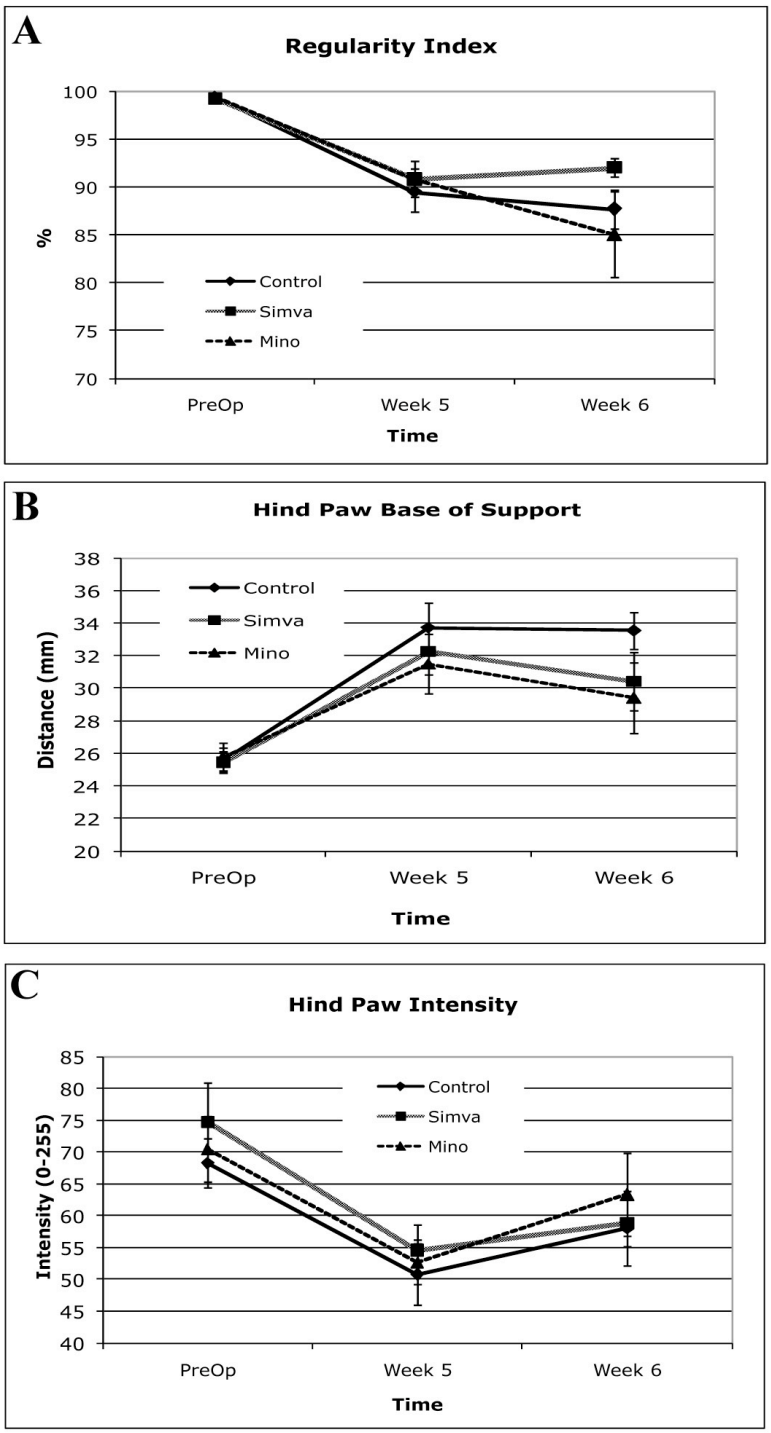


Figure 4.5.4: Histologic analysis of white and grey matter sparing at and around the injury epicenter.

(A) Representative microphotographs of spinal cord cross sections six weeks after SCI. (B) Quantification of white matter sparing revealed that neither simvastatin nor minocycline had a significant ($P<0.05$) neuroprotective effect as compared to the control treatment. (C) Adding the spared white matter over a distance of 1.6mm surrounding the injury epicenter also showed no significant differences. Similar analyses evaluating grey matter sparing, point-by-point (D) and spanning 1.6mm (E), revealed no neuroprotective effect from either drug.

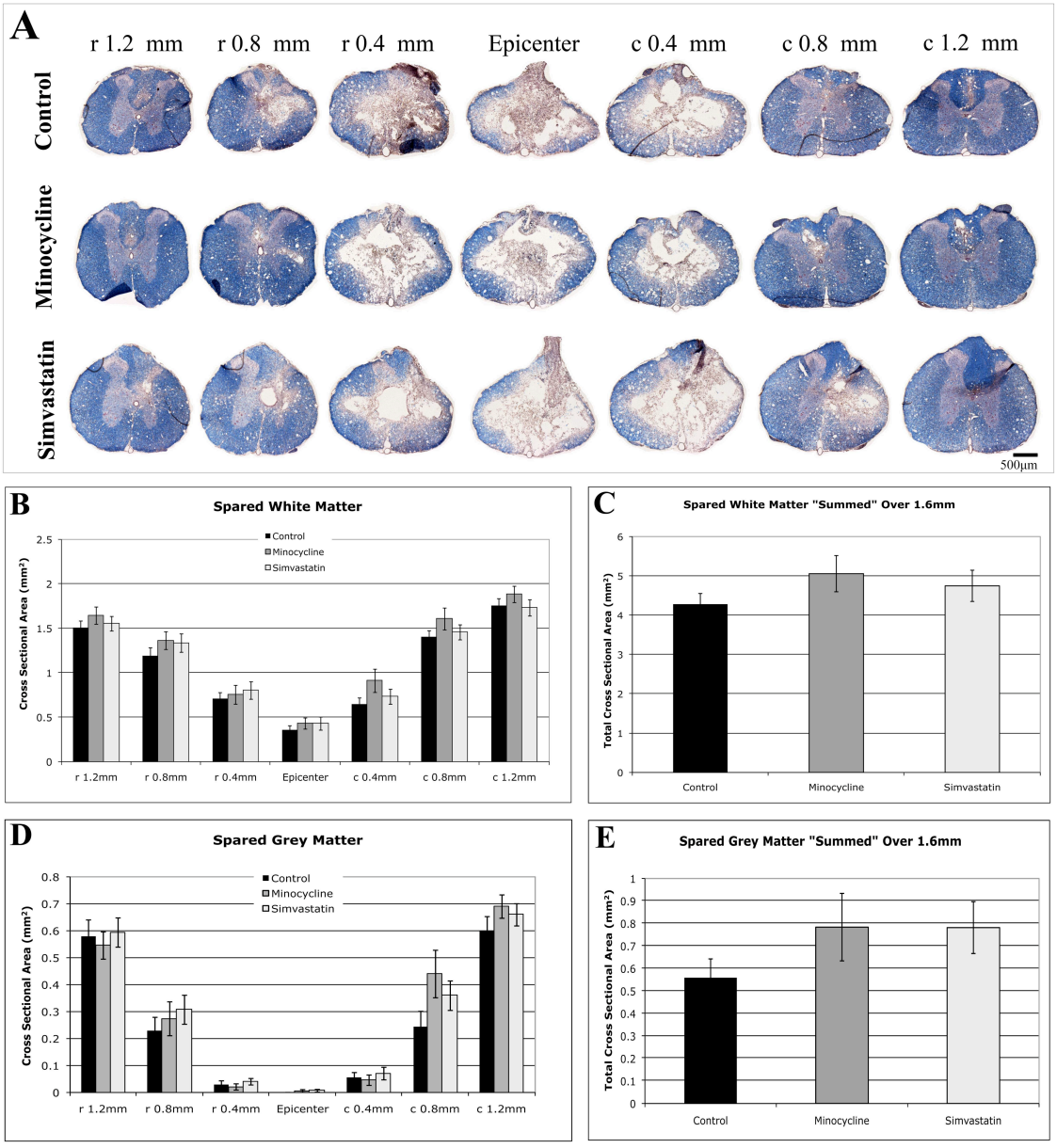
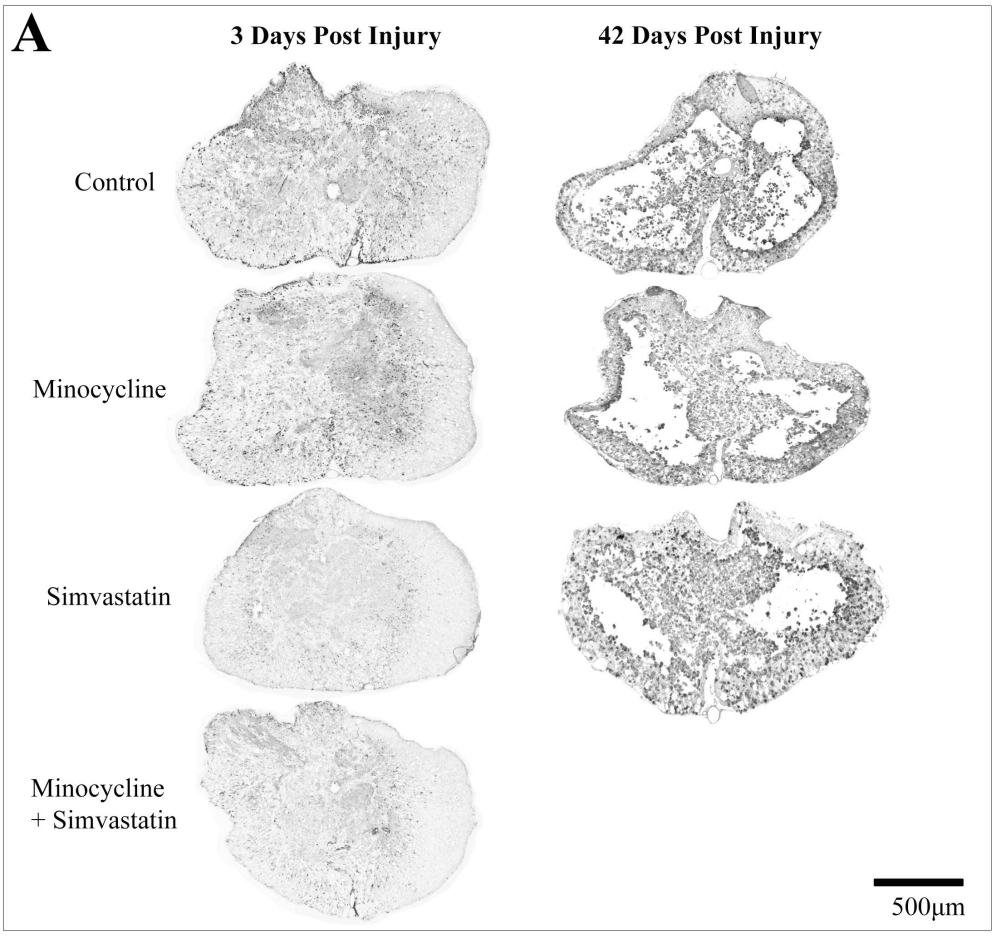
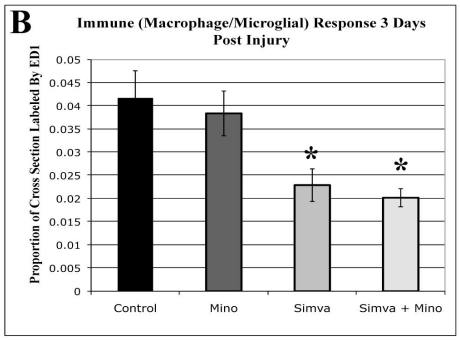
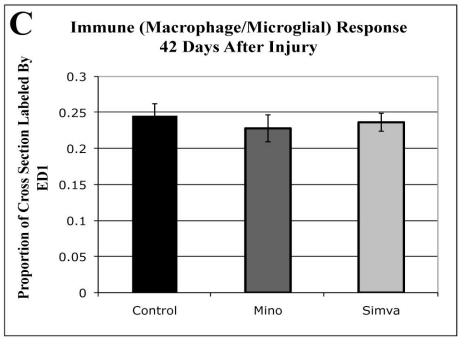


Figure 4.5.5: Histologic analysis of inflammation at the injury epicenter.

The activation of microglia and the presence of blood borne macrophages were quantified at the injury epicenter by immunostaining with an ED1 antibody. Representative images from the control, minocycline, and simvastatin treatment groups are presented (A). Microglial activation or macrophage presence is expressed as the proportion of ED1 positive area in the total area of spinal cord cross section. This analysis was performed 3 and 42 days post injury. (B) 3 days post injury, animals treated with simvastatin (alone or in combination with minocycline) significantly reduced (*, P>0.05) ED1 immuno-reactivity as compared to the control group. (C) At 42 days post injury neither drug influenced ED1 immuno-reactivity.







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CHAPTER 5

Conclusion

5.1 OBJECTIVES

Over the course of several experiments, I wished to evaluate the therapeutic efficacy of five neuroprotective agents for spinal cord injury (SCI). Erythropoietin, darbepoetin, atorvastatin, simvastatin, and minocycline have all previously demonstrated the potential to improve the outcomes of central nervous system (CNS) injury (Tables 1.5.1-1.5.4). Furthermore, these drugs are "safe to use" in humans since they are routinely prescribed by physicians for disorders unrelated to CNS injury. My overall objective was to evaluate them in an experimental model of SCI and compare them with each other in an attempt to find an optimal treatment. These attempts were made in order to discover a treatment that would not only have the greatest chances of success in a human clinical trial, but also one that would be quickly translated because an established drug safety profile.

A contusive injury using an Ohio State University (OSU) Impactor was applied to the rat spinal cord to produce a spinal cord injury that is analogous to the majority of those seen in humans[1]. This experimental model of SCI was used to perform a series of experiments that had the following objectives:

1. Determine if erythropoietin can attenuate secondary damage and increase neurological recovery in a clinically relevant model of spinal cord injury, and compare these results with darbopoietin, a derivative of erythropoietin.

2. Compare two pharmacologically different statins, simvastatin and atorvastatin, in their ability to attenuate secondary damage and increase neurological recovery in a clinically relevant experimental model of spinal cord injury.

3a). Enhance the neuroprotective effect of simvastatin in experimental spinal cord injury by extending its dosing regimen.
3b). Determine if the beneficial effect of simvastatin on experimental spinal cord injury can be augmented by concurrent minocycline administration.

5.2 RECAP OF RESULTS

5.2.1 The comparison of intravenously delivered darbepoetin and erythropoietin in their ability to improve outcomes after spinal cord injury

Erythropoietin and darbepoetin, a derivative of erythropoietin with an enhanced biological half-life, were shown to have no neuroprotective benefit as compared to the control treatment. Both drugs were delivered directly into systemic circulation by intravenous injection one hour after spinal cord injury. Injured animals were observed for 42 days and their motor and sensory recovery were evaluated. Unfortunately, we were unable to detect any differences between groups. Accordingly, histological analyses also showed no differences between groups, suggesting that neither drug protected the spinal cord from secondary pathology. Our results are at odds with those of others who have performed similar experiments with the same drugs. However, a closer look reveals that multiple differences exist in experimental design between our study and those performed in other labs. For instance, differences arise in the strain, weight and sex of the rats used and furthermore many different devices have been used to model SCI. My results are corroborated by a recent study by Pinzon et al[2] that attempted to reproduce the neuroprotective effects of erythropoietin in SCI. They performed a series of experiments to reproduce the very promising findings described by Gorio et al[3,4] and, surprisingly, were unable to generate the same results despite attempting to re-create the same experimental conditions[2]. They, like us, were unable to observe the beneficial effects of erythropoietin in SCI and suggested that the discrepancy between their results and what is reported in the literature is caused by slight differences in experimental design.

5.2.2 The comparison of orally administered atorvastatin and simvastatin in their ability to improve outcomes after spinal cord injury

Simvastatin, not atorvastatin, was shown to improve histological and neurological outcomes after experimental spinal cord injury. The two drugs belong to a family of popular cholesterol lowering drugs known as statins. Although they both are similar in their biological action, simvastatin was chosen because it has the desirable trait of being more lipophilic and therefore potentially more available to the CNS. Both agents were delivered to animals identically, through oral gavage one hour after injury. Treatment was continued for one week and animals were dosed once a day with 5mg/kg or 20mg/kg of atorvastatin or simvastatin respectively. 42 days after injury, it was determined that only simvastatin significantly improved

gross locomotor scores (BBB scale) and histological parameters. This is the first time, to our knowledge, that simvastatin has been shown to be efficacious for the treatment of experimental SCI. The inability of atorvastatin, which has demonstrated neuroprotective effects after experimental SCI[5,6], was attributed to differences in experimental procedure, such as the in length of drug administration, injury model, and gender of rats utilized. Our novel results with simvastatin encouraged us to perform further experiments with this drug to refine and maximize its beneficial effects.

5.2.3 The effect of long-term subcutaneous simvastatin treatment on experimental spinal cord injury and its comparison with intraperitoneal minocycline treatment.

Simvastatin treatment was extended to 42 days (compared to 7 days in the previous experiment) in hopes that this would maximize the benefit of using the drug. However, doing so did not improve the drug's beneficial effect, and rather, we did not observe a neuroprotective effect at all. Simvastatin treatment was also combined with 3 days of high dose minocycline treatment, to see if this would afford an additive or synergistic effect on neurological recovery. Unfortunately we were unable to evaluate this combinatory treatment since these two drugs appear to have a toxic drug interaction at the dosages utilized and induced significant morbidity and mortality. In this study we chose to deliver simvastatin subcutaneously to minimize the stress created on the animals by daily dosing. Treatment was started one hour after injury and continued at 20mg/kg for three days and 5mg/kg for the remainder of the experiment. Interestingly, simvastatin significantly decreased the microglial/macrophage inflammatory response 3 days after injury. Despite this robust anti-inflammatory effect, extended treatment of simvastatin did not have any effect on neurological recovery or tissue sparing 42 days after injury. These results, which differ from our previous study with simvastatin, are likely due to the differences in treatment method, duration, and dosage. Furthermore it is possible that long-term treatment negatively influenced reparative and protective functions of inflammation. Minocycline was delivered via intraperitoneal injection, with an initial dose of 90mg/kg followed by a 45mg/kg dose every 12 hours for 3 days. It too had no effect on neurological recovery and histological parameters. Numerous others have tested minocycline in experimental spinal cord injury and have reported that this agent has neuroprotective and anti-inflammatory effects – neither of which we were able to reproduce. We once again attribute our unexpected results to differences in experimental procedure.

5.3 DISCUSSION

As described throughout the introduction and the following manuscript chapters of this thesis, the drugs I chose to evaluate for the treatment of SCI are extensively supported by literature. They all reportedly have the ability to attenuate certain aspects of the secondary damage that occurs after CNS injury. Erythropoietin and darbepoetin are believed to reduce oxidative damage, excitotoxicity, and apoptotic cell death[7]. Atorvastatin and simvastatin have been recognized as immune system modulators, with an ability to decrease inflammatory mediated damage[8-10]. Similarly, minocycline has also been demonstrated to attenuate detrimental aspects of inflammation and reduce apoptotic cell death [11]. All these classes of drugs have been tested in experimental SCI and have, in some form or another, shown that they can improve neurological recovery[3,5,6,12-17] (Tables 1.5.1-1.5.4). In the experiments performed in this thesis, only a one-week regimen of simvastatin, delivered orally at 20mg/kg, improved outcomes after spinal cord injury. This is somewhat concerning, and raises questions regarding the efficacy of these agents and how much this efficacy is dependent upon experimental conditions with which they are tested.

It is becoming more and more evident that the effect of different pharmacological agents is not always consistently reproduced and slight differences in experimental procedure can influence whether a drug works or not[2] (Tables 1.5.1-1.5.4). Ideally, preclinical development of a therapy should be performed using an experimental model that most closely resembles human spinal cord injury, since the final goal is to find a treatment that will be beneficial to paralyzed humans. It remains difficult to say which experimental model or set of conditions most accurately portrays the sequence of pathological events that occurs in humans[1,18]. This is complicated by the fact that human injuries are extremely variable and because the differences between experimental animals and humans remain unclear[1,18-20]. Nevertheless, several injury models exist, for instance: compressive, contusive, laceration, and chemical. Each of these has a distinct and different primary injury mechanism[1,18]. Contusive spinal cord injury models are thought to best stimulate the biomechanics and neuropathology of human injury, however it has been said that no single model replicates all aspects of human spinal cord injury[1,18].

Most studies that have evaluated pharmacological agents with neuroprotective properties have done so using a contusive model (Tables 1.5.1-1.5.4). Contusive spinal cord injuries in animals are produced by two primary methods, by a weight drop device (ie. New York University, NYU impactor) or by a computer controlled electromechanical device (ie. Ohio State

University - OSU or the Infinite Horizon - IH impactor)[1,18].Our lab was the first to evaluate erythropoietin, darbepoetin, simvastatin, atrovastatin, and minocycline using the OSU impactor (Tables 1.5.1-1.5.4). Judging by the results we have obtained, it seems that the spinal cord injury induced by our model is less responsive to the biological action of these drugs. Despite the fact these agents have been efficacious in other contusive models, we were unable to reproduce the results of others.

Both the weight drop and electromechanical injury devices have been validated and used extensively by other labs studying SCI[1,18]. The two differ in their primary injury mechanisms. The weight drop injury is performed by dropping a weight from a set height. This device allows for the precise measurement of impact velocity, distance and rate of spinal cord compression, and the force applied to the cord. On the other hand, the injury caused by a computer controlled electromechanical impactor typically delivers an impact to the cord at a predetermined displacement. During this process, the force delivered to the cord and the velocity at which the impactor strikes the cord is recorded. Although the injury that these devices produce can be similar, many factors can vary depending on which settings are chosen. For instance, the speed at which the impactor strikes the cord, the amount of spinal cord that is displaced, and the force that is ultimately delivered can vary from one study to another.

In a laboratory setting, experimental injuries are tightly controlled and variability is kept to a minimum in a given study. In contrast, the injuries humans suffer from are extremely variable. They happen from different causes, at different spinal levels, and in different intensities. For instance, the number one cause of a spinal cord injury in humans is a motor vehicle accident. If an accident were to occur at 60 kilometers per hour (16.7 meters per second), an abrupt stop could deliver a fragment of bone into the spinal cord at a speed up to 16.7 meters per second. Of course, in this example the injury could vary depending on how fast the driver was initially traveling, the type of accident, the position the victim was in during the accident, and so on. Clearly it would be hard to imagine that a single human SCI will ever be reproduced in the exact same way. In our lab, the injuries were consistently delivered to the rat spinal cord at a maximum velocity of 0.31 meters per second, at a constant displacement (1.5mm), and at the same spinal level (T9/T10) in each rat. Despite keeping all these variables relatively constant, our model was still not sensitive enough to detect a neuroprotective effect from the drugs others have suggested as beneficial treatments for SCI.

The results that have been described in this thesis highlight a very important principle; differences in experimental procedure can dramatically influence the visible effect of a given

drug. The strain, sex, weight of the rats utilized, and biomechanical characteristics of the injury differ amongst studies (Tables 1.5.1-1.5.4). The time at which the drug was first administered and how frequently it is administered afterwards also differs amongst studies (Tables 1.5.1-1.5.4). If differences of this nature significantly influence a drug's neuroprotective efficacy in the tightly controlled experimental setting, then it is hard to envision how such a drug would be effective in the clinical setting where age, race, and injury characteristics, and time to intervention may vary enormously between patients. For a drug to successful in clinical trial, it is likely that it will need to have a consistent and robust effect that can be applied across many experimental paradigms.

With that said, there are several limitations to the widely used thoracic spinal cord injury model. Over the course of my experiments I have come to realize that there are many factors that determine the extent of neurologic injury deficit the animal procures. Although this correlates with the amount of viable tissue spared from injury, this relationship is not as strong as one would expect. Descending axonal tracts from the brain are largely responsible for the control of spinal motor systems. Yet the amount descending tracts that are preserved at the injury site does not necessarily determine how much neurological function a given animal will recover (Figure 1 and Figure 2). After a contusive thoracic injury, animals are typically paralyzed at first, however they tend to recover quite significantly over time, even though relatively little tissue is left at the injury epicenter. A substantial amount of this recovery is thought to occur from sprouting and plasticity which lets spared descending inputs control the central pattern generators that reside below the injury[21-23]. This plasticity in turn is largely influenced by many factors that are hard to control in the rat such as learning and activity[24].

Given the uncontrollable nature of our thoracic rat injury model, intergroup variability can severely hinder the ability of an experiment to detect a difference between a treatment and a control group. To evaluate the neuroprotective properties of a pharmacological agent, it may be useful to incorporate the use of an injury model that has a more direct relationship between white matter (ie. the number of axons directly injured) and neurological outcome. A recently described cervical hemi-contusion injury model may be more sensitive to the action of a neuroprotective agent since the amount of spared tissue correlates very well with neurological outcome[25] (Figure 2). Furthermore, injury to the grey matter of the thoracic spinal cord causes neurological deficits that are not easily identified. Conversely, grey matter injury at the cervical level causes neurological deficits that are clearly visible. For instance the loss of forelimb function would depend on injury to both grey matter and white matter. Lastly, a cervical injury model would

more accurately portray a human injury since the majority of human injuries occur in the cervical segments[19].

Overall, it still remains difficult to say which experimental paradigm will best evaluate a neuroprotective agent, or in other words, which paradigm stimulates a human injury most accurately. For a drug to work in the human setting, it will have to present a robust effect that can be applied over many injury models. The ability to work regardless of variations in injury type and severity or animal strain and gender will give that drug the greatest likelihood of success in the clinic. Currently there are not many options available for those who have had their spinal cord injured. This has created a sense of urgency in the community and is tempting many clinicians to test treatments that have been successful in animal experiments. Although treatments for human spinal cord injury are desperately needed, it will be valuable to make sure that experimental studies have been consistently reproduced. If the effect of a given drug cannot be consistently reproduced, it does not necessarily exclude the treatment altogether. Further work with it may be necessary to find an optimal treatment regimen. The experiments in this thesis suggest that further preclinical studies will be necessary before drugs such as erythropoietin, darbepoetin, simvastatin, atorvastatin or minocycline can be taken to clinical trial.

5.4 FIGURES

Figure 5.4.1: Tissue sparing and neurologic recovery.

Eight untreated animals were selected from an experiment conducted for chapter 4 of this thesis: "*The effect of long-term subcutaneous simvastatin treatment on experimental spinal cord injury and its comparison with intraperitoneal minocycline treatment"*. Representative images from the injury epicenter of the selected animals are displayed along with their respective scores on the BBB locomotor test and horizontal ladder test to measure footfalls. It is noted that the amount of spared spinal cord tissue (42 days post injury) does not necessarily correlate with neurological scores.

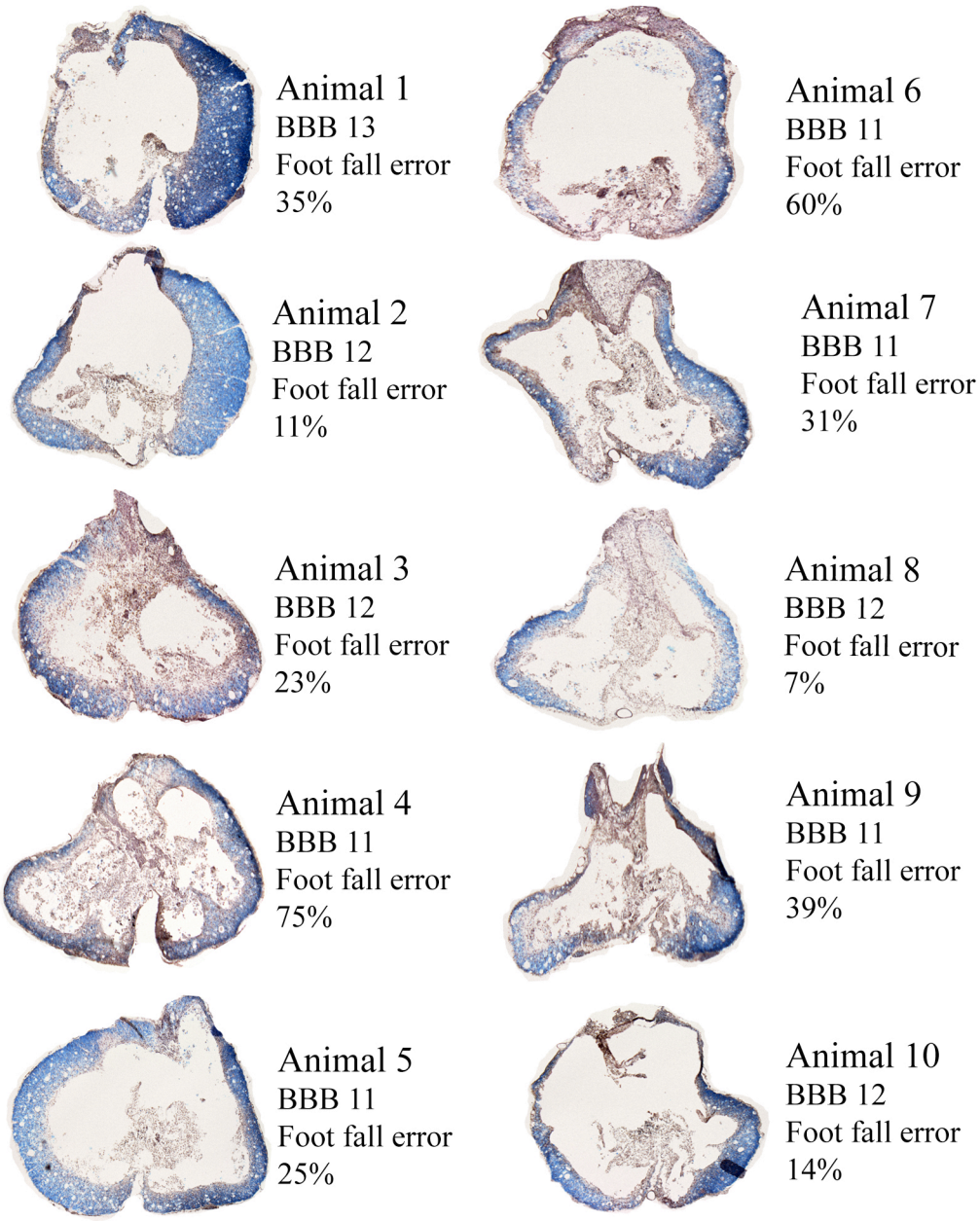
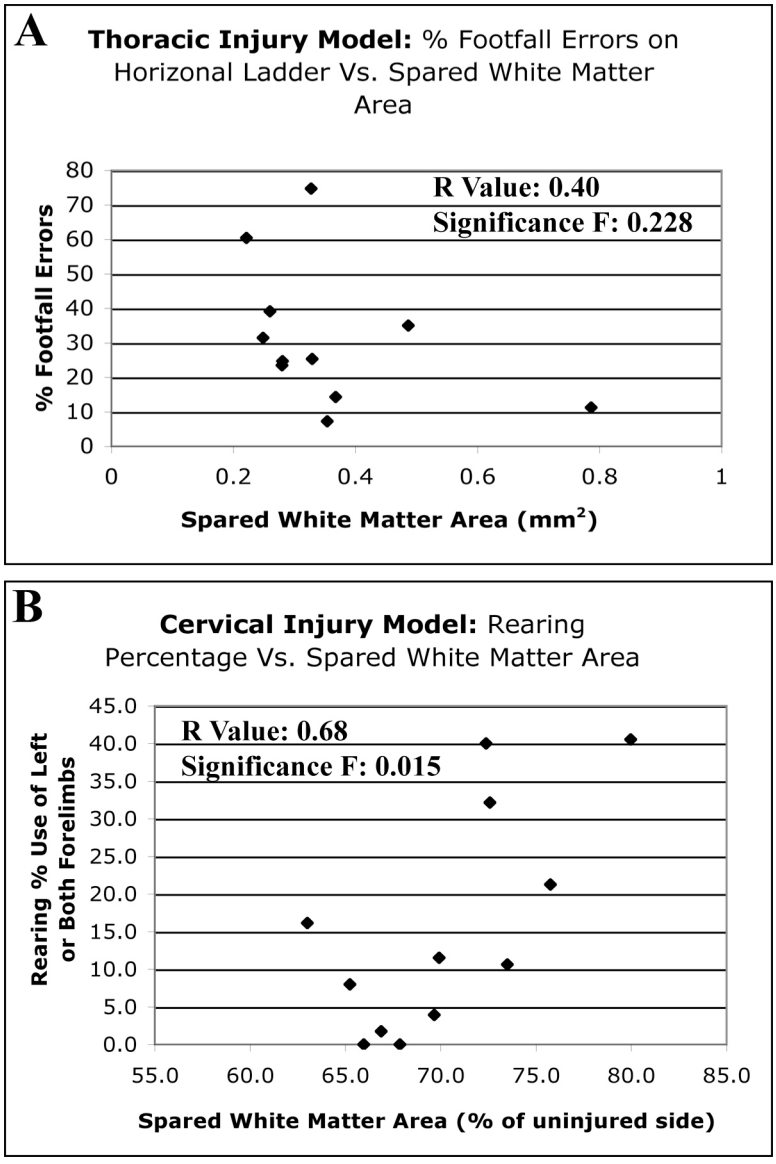


Figure 5.4.2: Correlations between tissue sparing and neurologic recovery.

A) Untreated animals were selected from an experiment conducted for chapter 4 of this thesis using the thoracic "contusion" injury model. The spared white matter area at the injury epicenter for each animal plotted against its percentage of footfall errors on the horizontal ladder test. A regression analysis revealed an R value of 0.40 with a statistical significance of 0.228 (F-test). B) Untreated animals were selected from an experiment in our lab using a cervical "hemi-contusion" injury model. The amount of white matter at the injury epicenter of the injured side was divided by the amount present on the uninjured side. This "percentage" of spared white matter was plotted against the same animals rearing percentage – a value that indicates usage of the paralyzed forelimb (left). A regression analysis revealed an R value of 0.68 with a statistical significance of 0.015 (F-test).



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APPENDIX

6.1 DETAILED METHODOLOGY

6.1.1 Injury Model and Surgery

Sprague-Dawley (SD) rats were obtained from Charles River Laboratories (Wilmington, MA) and housed 4 animals per cage. Environmental enrichment was provided for the animals in the form of a hollow plastic cylinder, which was kept in the cage at all times. Food was supplied ad libitum. All surgeries were performed under aseptic conditions and were conducted in accordance to UBC Animal Care guidelines. Prior to surgery, animals were shaved, and the skin was sterilized with Betadine and alcohol. All surgical instruments were autoclaved before use and sterilized with a bead sterilizer between uses during surgery. A midline incision was made at the thoracic region and the skin was retracted. Musculature was cut and separated to expose the T9/T10 thoracic region of the spinal column. A 3mm laminectomy was performed here (T9/T10) with a fine pair of rongeurs and care was taken to leave the dura intact and unharmed. The OSU impactor device was used to injure the spinal cord. The dorsal process of the 8^{th} and 10^{th} thoracic vertebrae was held with modified allis clamps while 50% of the body weight was supported from underneath. The impactor was lowered onto the spinal cord until it touched the spinal cord and a built in sensor allowed the measurement of a "preload force" of 0.2 kdyne onto the dura. Thereafter, an abrupt advance of the impactor (1.5mm in 1msec, with a maximum speed of 300mm/sec) produced a moderate and reproducible spinal cord injury. The clamps were then removed and the wound closed by rejoining the skin with metal clips (Michel). The OSU device calculates and reports the overall force delivered to the spinal cord at a given moment during the course of the contusion. Animals were excluded if the peak force fell outside the range of 200-260 kdynes to keep the severity of spinal cord injury relatively consistent within groups and the experiment.

6.1.2 Behavioral Assessments

BBB Locomotor Test: Open-field locomotor testing was performed by two blinded examiners on days 2, 7, 14, 28, 35, and 42 days post-injury using the Basso, Bresnahan, and Beattie (BBB) locomotor score[1]. Briefly, the BBB scale ranges from 0 to 21 and scores gross locomotor ability. Scores from 0 to 8 indicate improvement of hip, knee and ankle movement, 9 to 14 indicate improving stages of weight support, stepping, and coordination[1]. Finally, finer

details in locomotion are scored by 15 to 21, these levels indicate toe clearance, paw position, and tail position[1]. Hence, an animal with completely paralyzed hindlimbs would be scored as a 0 and a healthy animal would be scored as a 21. The finer details of locomotion (represented by BBB 15 to 21) cannot be taken into account until a rat has reached consistent coordination of weight supported plantar stepping (BBB 14). Rats can however recover these finer traits before they are coordinating their steps. Therefore an additional subscore was used to evaluate the observation of normal paw positioning, toe clearance, and tail lift before the recovery of coordinated stepping. A scale ranging from 0-13 represented recovery of these traits. A score of 13 signified parallel paw positioning at initial contact and lift off, consistent toe clearance, and a raised tail during locomotion[2]. All open-field locomotor testing was performed under the same conditions and at the same time period on each scheduled testing date.

Catwalk Gait Analysis: The CatWalk gait analysis system was recently developed to provide an enhanced tool to measure functional outcomes after spinal cord injury[3]. It consists of an automated walkway that records the footprints of a small animal such as a rat as it walks across. The entire sequence of steps in one "run" from end to end is recorded and saved as a digital file by a computer. The accompanying computer program can then be used to analyze locomotion in great detail, and to give several different measurements of gait. We used this system to follow the motor recovery of our injured animals at 5 and 6 weeks post injury. During these sessions, 5 runs were collected per animal and averaged to obtain an accurate representation of a given animals locomotor ability. The computer analysis program was then used to produce 3 outcome measures of specifically chosen gait parameters: regularity index, average hindpaw base of support, and average hind paw intensity[3].

The *regularity index* is based on the concept of regular step patterns and serves as a relative measure of coordination. The index decreases after thoracic spinal cord injury.

Hindpaw base of support measures the width of the hindpaws during locomotion. This distance increases after spinal cord injury to compensate for an unstable gait.

Hind paw intensity is a measure of the signal that is created when the animal steps on the walkway. This allows a relative value of how much pressure or weight the injured

animals are supporting on a particular paw. The amount of weight supported by a hindpaw decreases after injury to the thoracic spinal cord.

6.1.3 Histologic Assessments

Immunohistochemical staining: Spinal cords were sectioned axially at 20μm with a cryostat-microtome and sections were mounted on Superfrost Plus slides (Fisher). Since the sections are frozen and stored at -70 degrees Celsius, they were thawed at room temperature for 2-3 hours before being washed three times in 0.01M PBS. Sections were then permeabalized with 0.1% TX 100 in 0.01 PBS for 1 hour and washed with PBS again (2-3 times). Next, sections were blocked with 10% normal donkey serum for one hour. This was immediately followed by an overnight incubation with the chosen primary antibody (in 0.1% TX 100 in 0.01PBS). The next day, sections were washed three times with 0.01M PBS and then incubated with a florescence-conjugated (donkey) secondary antibody (in 0.01PBS) for an hour. Lastly, sections are washed three times with 0.01M PBS and cover-slipped with fluoro-mount mounting medium.

In addition, a DAB (3,3'-Diaminobenzidine) staining protocol was used to eliminate the miscalculation of auto-florescent background cells as immune cells, a problem that arises with florescent immuno-staining. Spinal cord sections were washed with PBS, Triton X-100, and with the appropriate normal serum to block nonspecific binding. Endogenous peroxidase activity in spinal cord tissue was quenched with 0.3% hydrogen peroxide in methanol. The tissue was then incubated overnight at room temperature with a monoclonal mouse ED1 antibody (1:500, Serotec). Sections were then incubated the next day with a secondary antibody conjugated to biotin for one hour at room temperature. This was followed by a treatment with ABC complex (Vector Laboratories) for one hour and, lastly, antibody labeling was visualized by applying DAB substrate for 5 minutes.

Measurement of the inflammatory response: The inflammatory response was measured by determining the relative proportion of the spinal cord cross section that was stained by the ED1 antibody (1:500, Serotec). This allows for the quantification of macrophages and activated microglia in the spinal cord (as described by Popovich et al[4]). However to prevent the miscalculation of non-specific background signal on the section, threasholding was applied to isolate ED1 labeling. In our experience, the background signal from a particular animal can be influenced by numerous factors that are difficult to control, for instance perfusion and staining

abnormalities. Therefore a formula was developed that produced a threshold value proportionally based on the average intensity of non-ED1 stained white matter tissue on a particular section. The threshold for each section was calculated by subtracting 90 intensity units (0-255) from the average intensity of non-inflammatory tissue, which was determined by sampling spared white matter (ImageJ software). This allowed a variable threshold to be applied to each section, which was based on the sections inherent signal intensity. Thereafter, the area labeled with ED1 on the spinal cord cross section was calculated (ImageJ software) and divided by the total cross sectional area to give a "proportion". 3 spinal cord cross sections (per animal), from the epicenter of injury, were quantified and averaged to obtain a more accurate sample from each animal.

6.2 UBC RESEARCH ETHICS BOARD CERTIFICATES OF APPROVAL

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1754

Investigator or Course Director: Brian Kwon

Department: ICORD

Animals:

Rats Sprague-Dawley 596

Start Date: **January 2, 2005** **Approval Date:** **January 15, 2008**

Funding Sources:

Funding Agency:	Wings for Life Spinal Cord Research Foundation
Funding Title:	The preclinical evaluation of drug combinations as a neuroprotection strategy for acute spinal cord injury
Funding Agency:	Scoliosis Research Society
Funding Title:	Neuroprotection for acute spinal cord injury: the preclinical evaluation of drugs that are currently used in human non-spinal applications
Funding Agency:	Rick Hansen Man In Motion Foundation
Funding Title:	Cerebrospinal Fluid Drainage and Cytokine Analysis in the teratment 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
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

THE UNIVERSITY OF BRITISH COLUMBIA

Cody Mann

has successfully completed the online training requirements of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program

Chair, Animal Care Committee

Veterinarian

Certificate #: 1698

Date Issued: May 23, 2006

6.3 REFERENCES

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