GLUTAMINE:
A NOVEL AND POTENT THERAPEUTIC FOR ACUTE SPINAL CORD INJURY

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By

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ABSTRACT

Spinal cord injury occurs at a rate of 11.5 - 53.4 per million in developed countries with great emotional and financial consequences. The damage caused by the initial injury is followed by secondary damage, a complex cascade of mechanisms including ischemia, oxidative stress, inflammation and apoptosis. Although nothing can be done to reverse the initial damage to the spinal cord once it occurs, the secondary damage can be targeted by therapeutics to improve recovery. Following injury, concentrations of the potent antioxidant glutathione (GSH) are decreased in the spinal cord which potentiates mechanisms of secondary damage. In an attempt to maintain the GSH concentrations, the non-essential amino acid glutamine was tested as it was shown to increase GSH concentrations both in vivo and in vitro. Glutamine is being used extensively in clinical research in an expansive number of physiological and pathological conditions including brain trauma.

To examine the therapeutic potential of glutamine after spinal cord trauma, two compression injury models, the modified aneurysm clip and the modified forceps, were used to induce an injury in male Wistar rats. We have demonstrated the ability of glutamine treatment (1 mmol/kg), given 1 hour after a 30 g aneurysm clip injury to increase GSH not only in whole blood samples but within the spinal tissue at the site of injury. Increasing GSH in this way also resulted in improved locomotor scores and maintenance of white matter tissue at the injury epicenter. Experiments using the forceps model were then performed to determine if the potency of glutamine treatment would be carried over to a different model and at a variety of severities. Glutamine, again,

demonstrated the ability to improve maintenance of whole blood GSH, locomotor scores and tissue histology.

In our experiments, glutamine has proven to be a potent therapeutic for spinal cord injury with an effect that is matched by few compounds currently being studied and well exceeding the standard therapeutic, methylprednisolone. Given the breadth of knowledge regarding the effects of glutamine clinically in numerous paradigms and the potency of the therapeutic effect seen in these studies, we believe that glutamine is fit for clinical trial and has a high potential for success.

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DEDICATION

To my parents, Carolyn and Joe Rigley, and my husband Peter MacDonald,

I am where I am, and who I am, because of you.
Thank you is not enough.

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LIST OF ABBREVIATIONS USED

ANOVA	analysis of variance	LFB	luxol fast blue
ATP	adenosine triphosphate	MPO	methylprednisolone
BBB	Basso beattie Bresnahan	µl	microliter
BBB	blood brain barrier	µm	micrometer
CNS	central nervous system	µm	micromole
CSF	cerebral spinal fluid	mg	milligram
CST	corticospinal tract	ml	milliliter
cys	cysteine	mM	millimolar
cys-cys	cysteine-cysteine (cystine)	mm	millimole
DTNB	5,59-dithio-bis(2-	M	molar
	nitrobenzoic) acid	MCB	monochlorobimane
EDTA	ethylenediaminetetraacetic	nm	nanometer
	acid	nm	nanomole
GABA	γ-aminobutyric acid	NFkB	nuclear factor kappa B
γ GCL	glutamate cysteine ligase	NMR	nuclear magnetic resonance
GLN	glutamine	OTZ	L-2-oxothizolidine-4-
glu	glutamate		carboxylate
gly	glycine	PFA	paraformaldehyde PBS
GS	glutathione synthase		phosphate buffered saline
GSH	glutathione	ROS	reactive oxygen species
g	gram	RST	rubrospinal tract
HPLC	high pressure liquid	SCI	spinal cord injury
	chromotography	TCA	tricarboxylic acid
IP	intraperitoneal	U	unit
kg	kilogram	UV	ultraviolet

CHAPTER 1.0

INTRODUCTION

1.1 Spinal cord injury

1.1.1 Prevalence and cost of spinal cord injury

Spinal cord injury (SCI) occurs at a rate of 40 per million in the United States (NSCISC, 2006) with a range of 11.5 - 53.4 cases per million within developed countries (Sekhon and Fehlings, 2001). In a study focused on the patient population at the London Health Sciences Center (Ontario, Canada) between 1997 and 2000 the mean age was found to be 42.2 years and a ratio of 3:1 male to female (Pickett et al., 2006). The most common causes of SCI were motor vehicle accidents (35%) and falls (31%) (Pickett et al., 2006).

The highest incidence of SCI is seen between 10 - 40 years of age (Sekhon and Fehlings, 2001; Pickett et al., 2006) which is during the patient's prime years of earning. Employment rates of people who were pediatric SCI patients was 54% compared to 84% for the general public (Vogel et al., 1998). In the United States, the average costs the first year following injury have been calculated at $233,000 - $295,000 with recurring annual costs between $17,000 - $33,000 and lifetime cost of $630,000 - 970,000 (DeVivo, 1997). It has been estimated that the average annual costs for SCI management in the United States is approximately $7.8 billion dollars (DeVivo, 1997) and these costs do not take into account lost wages or decreased earning potential. Clearly, aside from the obvious personal strain a SCI injury would cause the patient and family, there is a

substantial financial burden placed on society by SCI which could, at least partially, be alleviated by effective therapeutics.

1.1.2 Phases of SCI

1.1.2.1 Primary damage

The damage cause by SCI is described by the primary and secondary phases. The primary phase, involving the initial mechanical damage to the spinal cord tissue by contusion, compression, laceration, shearing or a combination, is thought to comprise only 10% of the overall injury to the tissue (Young et al., 1982). SCI can occur as a result of displaced bone fragments or foreign objects which cause laceration or transection of the spinal cord (Tator, 1983). Hyperflexion or hyperextension of the vertebrae can also result in SCI due to the shearing within the spinal cord (Silberstein and McLean, 1994). The most common primary mechanisms of SCI are fracture dislocation and burst fractures (Tator, 1983; Sekhon and Fehlings, 2001; Pickett et al., 2006).

A fracture dislocation occurs when a portion of the vertebrae is fractured and moves into the spinal canal causing damage to the cord. This type of injury is sub-classified according to the direction of the dislocation, namely anterior, posterior or lateral, with anterior being the most common (Tator, 1983). Fracture dislocation injuries result in an initial contusion of the spinal cord, followed by a sustained compression (Tator, 1983). Burst fractures occur due to the complete collapse of the vertebral body which causes the margin of the body to extend. Bony fragments then often enter the spinal canal causing contusion, compression and possibly laceration of the spinal cord. Tator et al. (1983) found that both these injury types most commonly result in complete

SCI, meaning a total loss of sensory information and motor control distal to the injury site.

1.1.3.2 Secondary damage

Many cells appear to survive the initial injury but degenerate over time due to the cascade of tissue destructive mechanisms which follow (Tator and Fehlings, 1991). The secondary phase which begins immediately after injury and can last for days, weeks or months is a highly complex process involving numerous inter-related mechanisms including ischemia, inflammation, oxidative stress and excitotoxicity (Kakulas, 1987; Anderson, 1992; Juurlink and Paterson, 1998; Tator, 1998; Di Giovanni et al., 2003; Beattie, 2004; Kwon et al., 2004; Norenberg et al., 2004; Profyris et al., 2004; Xu et al., 2005; Baptiste and Fehlings, 2006; Hagg and Oudega, 2006; Keane et al., 2006; Trivedi et al., 2006).

1.1.3.2.1 Ischemia

Spinal cord ischemia occurs following SCI and, if not treated, progressively increases (Fehlings et al., 1989) and can persist for 24 hours in the rat (Rivlin and Tator, 1978b). Many mechanisms are responsible for the ischemia including vasospasms (Tator and Fehlings, 1991), endothelial swelling or damage (Dohrmann and Allen, 1975) and hemorrhages (Wallace et al., 1986). Disruptions in spinal cord blood flow appear to predict the tissue disruption pattern seen, namely, ablation of gray matter with maintenance of the peripheral white matter preferentially in a contusion injury (Kloos et al., 2005).

Following SCI in dogs and rabbits, in the peripheral white matter, which is typically the first tissue spared after injury, blood flow returns to normal within 5 minutes and has normal values for 24 hours (Fairholm and Turnbull, 1970). The central gray matter, which appears to be the most vulnerable to SCI, undergoes a substantial decrease in blood flow which is maintained for at least 24 hours with numerous hemorrhages occurring (Fairholm and Turnbull, 1970; Sandler and Tator, 1976; Wallace et al., 1986). Wallace et al. also found that white matter damage was especially severe in areas adjacent to gray matter hemorrhage which are associated with increased immune cell infiltration and inflammation.

1.1.3.2.2 Inflammation

The spinal cord is, under physiological conditions, an immune privileged organ and only when there is a disruption to the spinal cord blood barrier are non-endogenous immune cells present. As was mentioned above, hemorrhage occurs frequently within the gray matter of the spinal cord and this is an important route for the passage of immune cells into the spinal tissue. Although the initial inflammatory response has been shown to be detrimental due to an overabundance of reactive oxygen species produced (Fleming et al., 2006; Trivedi et al., 2006), the clearance of cellular debris by immune cells to allow for regrowth is essential to the reparatory process (Schwartz and Yoles, 2006; Ziv et al., 2006). Research examining the importance of the immune response to both destructive and beneficial processes has been the subject of many studies and reviews (Popovich et al., 1999; Bethea and Dietrich, 2002; Popovich et al., 2002; Profyris et al., 2004; Jones et al., 2005; Trivedi et al., 2006; Donnelly and Popovich, 2008; Popovich and Longbrake, 2008). The difference between the beneficial and

detrimental appears to be in differentiating the beneficial and detrimental inflammatory responses following SCI.

The early response, which is considered detrimental, begins within hours of injury and is characterized by the beginning of the neutrophil influx (Bethea, 2000). Neutrophils are detected within the spinal cord within 4 hours after injury, peak at 12-24 hours and disappear 5 days post injury (Taoka et al., 1997; Fleming et al., 2006). Injury severity has been correlated to the proportion of neutrophil infiltration (Xu et al., 1990) as a large number of neutrophils in the tissue is associated with 'by-stander' tissue damage (Taoka et al., 1997). Neutrophils produce potent reactive oxygen species (Harlan, 1987) and have been demonstrated to have a role in ischemia/perfusion injury (Hernandez et al., 1987). Additionally, neutrophil depletion has been found to be neuroprotective following injury (Beril Gok et al., 2007).

The late phase of the inflammatory response is characterized by influx of macrophages whose numbers peak within 2-3 days (Bethea, 2000) but can remain for weeks or months after injury (Popovich et al., 1997; Carlson et al., 1998). The possible neuroprotective effects of the immune system are focused on the late phase of the inflammatory response and the role of macrophages in the trophic support for the injured tissues (Banati and Graeber, 1994; Nimmerjahn et al., 2005). Autologous macrophages are now being studied as a therapeutic intervention for SCI (Rapalino et al., 1998; Schwartz et al., 1999; Schwartz and Yoles, 2006).

1.1.3.2.3 Oxidative stress

Of the many mechanisms affecting the amount of secondary damage after SCI, one of the key factors is the pairing of oxidative stress (Aksenova et al., 2002; Bao and

Liu, 2002; Xu et al., 2005) and resultant inflammation (Jones et al., 2005; Fleming et al., 2006). In addition to neutrophils, microglia and macrophages produce the reactive oxygen species superoxide anion and nitric oxide which can combine to produce peroxynitrite, a highly reactive oxidant (Liu et al., 2000; Chatzipanteli et al., 2002). The involvement of oxidative stress in the development of neurotrauma is well documented and has been extensively reviewed (Juurlink and Paterson, 1998; Juurlink et al., 1998; Tan et al., 1998; Juurlink, 1999; Shohami et al., 1999; Christen, 2000; Schulz et al., 2000; Tyurin et al., 2000; Chan, 2001; Kamencic et al., 2001; Aksenova et al., 2002; Gilgun-Sherki et al., 2002; Lovat and Preiser, 2003; Kayali et al., 2005).

Lipid peroxidation is induced by oxidative stress following SCI (Springer et al., 1997a; Baldwin et al., 1998) and has numerous detrimental effects including: increased permeability of membranes (Subramaniam et al., 1997), decreased ATPase activity (Rauchova et al., 1995) and the production the pro-inflammatory compounds isoprostanes (Liu et al., 1998) and isoleukotrienes (Harrison and Murphy, 1995) as by-products. Oxidative stress and inflammation are closely linked and each can promote the other. Increased oxidative stress can cause the activation of nuclear factor kappa B (NFκB), a pro-inflammatory transcription factor (Flohe et al., 1997). NFκB will increase the inflammatory response resulting in reactive oxygen species (ROS) formation by immune cells which perpetuates the inflammation/oxidation cycle.

1.1.3.2.4 Glutamate-mediated excitotoxicity

Other metabolic byproducts of lipid oxidants are strong oxidants which can alter ion homeostasis (Mark et al., 1997), mitochondrial respiration (Keller et al., 1997; Picklo et al., 1999) and glutamate uptake (Keller et al., 1997; Springer et al., 1997b).

The ion gradient homeostasis is critical for maintenance of the calcium gradient across the cell membrane which, when disrupted, can lead to increased intracellular calcium concentrations. An increase in intracellular calcium resulting in increased release of glutamate into the synaptic cleft. Regulation of glutamate concentrations in the extracellular space is critical for the normal function of neurologic tissue. Excess glutamate in the extracellular space can lead to excitotoxicity causing dysregulation of calcium, increased reactive oxygen species formation and cell death (Perez Velazquez et al., 1997).

1.2 Therapeutic approaches

1.2.1 Standard clinical care following SCI

The current acceptable treatments for SCI involve stabilization of the vertebral column to prevent further mechanical damage to the cord, support of spinal cord blood perfusion and the administration of the steroid methylprednisolone (MPO). SCI is exacerbated by hypoperfusion as this leads to ischemia and its attendant problems. Maintenance of mean arterial blood pressure above 85 mm Hg has been shown to be effective in improving clinical outcome (Vale et al., 1997). The current clinically accepted intervention for combating oxidative stress and inflammation following SCI is the administration of the steroid MPO.

The support for the use of MPO began following the publication of the NASCIS II trial when there were improvements seen following a post-hoc test on data from patients that were treated within 8 hours of their injury (Bracken et al., 1990). The authors of a recent analysis of the literature conclude that there is no evidence that MPO administered following SCI has any therapeutic effect (Sayer et al., 2006) which has

been discussed previously by others (Nesathurai, 1998; Hurlbert, 2000; Hurlbert and Moulton, 2002). Other studies contradict that statement indicating that MPO gives mild or moderate results (Liu and McAdoo, 1993; Behrmann et al., 1994; Taoka et al., 2001; Takami et al., 2002; Weaver et al., 2005; Ates et al., 2006) but there is evidence of adverse side effects, particularly when MPO is given in high doses (Bracken et al., 1984).

Despite the variety of methods and models, few therapeutic strategies developed in the laboratory have been found to be effective clinically. In addition to species differences, one proposed explanation for this is the inaccuracy of the spinal model in mimicking the complexity of clinical SCI (Tator, 2006).

1.2.2 Potential of antioxidant/anti-inflammatory strategies

The use of antioxidants as therapeutic compounds is logical given the role of ROS in not only oxidative damage but inflammation and excitotoxicity following SCI. Two of the better known antioxidants, vitamins C and E have been studied for therapeutic potential with findings of improved blood flow and decreased lipid peroxidation (Hall et al., 1989; Iwasa et al., 1989; Taoka et al., 1990; Katoh et al., 1996; Wang et al., 2006). In addition, many other antioxidant compounds have shown to be moderately beneficial (Kaptanoglu et al., 2002; Hillard et al., 2004; Kayali et al., 2005; Sharma et al., 2006). Previous research from our laboratory using a procysteine compound and the flavonoid quercetin (Kamencic et al., 2001; Schultke et al., 2003) demonstrated substantial improvements in both locomotor function and tissue sparing at the site of injury, well beyond that of the previously mentioned works. Although many compounds have been studied in the laboratory setting, few have made a successful

transition to clinical trials with maintained potency. In order to be effective across injuries and species a number of issues should be considered, such as time of administration, ability to cross blood brain barrier and the similarity of metabolic mechanisms between species (Gilgun-Sherki et al., 2002).

The focus of this project has been to develop a treatment for SCI that will help the body compensate for the altered metabolic requirements following injury and which can be utilized by multiple organ systems. The aim is not only to improve oxidative stress but overall bodily function. We tested the effect of glutamine to decrease oxidative stress and improve recovery following SCI.

1.3 Glutamine

Glutamine has been studied and reviewed extensively in the clinical setting in this context (Buchman, 2001; Grimble, 2001; Young and Ajami, 2001; Neu et al., 2002; Garcia-de-Lorenzo et al., 2003; Newsholme et al., 2003b; Burrin and Davis, 2004; Melis et al., 2004; Wischmeyer, 2006, 2008). Although traditionally classed as a nonessential amino acid, there is a current consensus to reclassify glutamine as 'conditionally essential' in states of physiological stress (Lacey and Wilmore, 1990). Glutamine concentrations have been demonstrated repeatedly to be decreased following many types of trauma (Engel et al., 2003), burns (Parry-Billings et al., 1990; Gore and Jahoor, 1994), surgery (Blomqvist et al., 1995; Hammarqvist et al., 1996), and poor nutrition states (Van Der Hulst et al., 1994). Additionally, following SCI, plasma glutamine concentrations have been shown to decline in humans (Rogeri and Rosa, 2005) and in both plasma and muscle in rats (Tanhoffer et al., 2007). Decreases in plasma glutamine have been linked to immunodepression (Parry-Billings et al., 1990), increased muscle

catabolism (Kuhn et al., 1999) and decreased GSH content in muscle (Flaring et al., 2003).

Supplementation with glutamine has proven beneficial for a wide variety of conditions including gastrointestinal surgery or trauma (Wilmore, 2001), lethal hepatic injury (Hong et al., 1992), brain injury (Falcao de Arruda and de Aguilar-Nascimento, 2004), adult burn victims (Garrel et al., 2003), multiple trauma (Houdijk et al., 1998; Houdijk and van Leeuwen, 2000; Bakalar et al., 2006; Dechelotte et al., 2006), bone marrow transplant (Brown et al., 1998; Schloerb and Skikne, 1999; Coghlin Dickson et al., 2000; da Gama Torres et al., 2008), cardiac muscle ischemia (Khogali et al., 1998) and radio- and chemotherapy (Rouse et al., 1995; Yoshida et al., 2001; Savarese et al., 2003). The majority of beneficial effects of glutamine treatment appear to be in the mild to moderately ill, as studies examining critically ill patients however, have shown no positive influence on length of hospital stay (Kumar et al., 2007), infectious morbidity (Schulman et al., 2006) or mortality (Hall et al., 2003; Schulman et al., 2005; Kumar et al., 2007).

1.3.1 Synthesis, transport and metabolism of glutamine

Sixty percent of the free amino acid concentration in the body is glutamine (Darmaun et al., 1986), most of which is synthesized within the skeletal muscle (Welbourne, 1987), lungs (Welbourne, 1988) or adipose tissue (Frayn et al., 1991). The liver and kidney also play a role in glutamine synthesis but mainly in instances of increased glutamine utilization (Damian and Pitts, 1970; Gebhardt and Mecke, 1983). Glutamine is formed in an ATP-dependent manner with the ligation of ammonia to glutamate catlaysed by glutamine synthase (Krebs, 1935). Glutamine is absorbed from

food in the intestine but increases in the pro-inflammatory interleukin-1 decreases glutamine uptake (Austgen et al., 1992). Glutamine has numerous transporters throughout the body and, unlike glutamate, is able to cross the blood brain barrier. The major transporters for glutamine are Na^+-dependent system N transporters which have been found in human and rat tissues (Kilberg et al., 1980; Hundal et al., 1987; Tamarappoo et al., 1997; Nakanishi et al., 2001).

It is believed that glutamine is used by all cells for the synthesis of other amino acids, purines and pyrimidines but the overall use of glutamine for these purposes comprises less than 5% of the glutamine (Curthoys and Watford, 1995). Although it was initially thought that the intestinal mucosa was the largest consumer of glutamine as an energy source, it appears as though it only partially oxidizes glutamine and the end products are then used by other tissues (Watford, 1994). The immune system is now thought to be one of the largest glutamine consumers (Newsholme and Calder, 1997; Kew et al., 1999; Grimble, 2001; Newsholme, 2001; Newsholme et al., 2003b; Newsholme et al., 2003a; Bistrian, 2004) especially during lymphocyte proliferation as the rate of consumption increases 10-fold (Brand et al., 1986).

In the central nervous system (CNS) glutamine can be metabolized to produce both an excitatory (glutamate) and an inhibitory (γ-aminobutyric acid (GABA)) neurotransmitter (Peng et al., 1993; Rae et al., 2003; Waagepetersen et al., 2003). One of the key roles of glutamine in the CNS appears to be within the glutamine/glutamate cycle which consumes a significant portion of the energy derived from glucose in the cortex (Shulman and Rothman, 1998; Rothman et al., 1999). The cycle describes the uptake of glutamate from the extracellular space into astrocytes which will convert it to glutamine by glutamine synthase. The glutamine is then transferred back to the pre-

synaptic neuron which can convert it back to glutamate using the enzyme glutaminase thus completing the cycle (Behar and Rothman, 2001). The cycle is believed to have a neuroprotective effect in states of physiological stress by decreasing extracellular glutamate concentrations and inhibiting excitotoxic pathways (Gras et al., 2006). Central to this theory is the maintenance of glutamate uptake by astrocytes which can be inhibited by oxidative stress (Springer et al., 1997a; Rao et al., 2003).

1.3.2 Mechanisms of action of glutamine

One of the reasons for the potency and versatility of glutamine as a treatment is the multifaceted nature of its effects. Glutamine functions metabolically as a nitrogen donor for protein synthesis (Zalkin and Smith, 1998), a carbon donor for the tricarboxylic acid cycle (Tildon and Roeder, 1984; Tildon et al., 1985), an essential energy source for proliferating immune cells (Ogle et al., 1994; Newsholme and Calder, 1997; Kew et al., 1999; Chang et al., 2002; Peng et al., 2006a) and a substrate for ammoniagenesis (Halperin and Bun-Chen, 1987), gluconeogenesis (Nurjhan et al., 1995; Stumvoll et al., 1998; Stumvoll et al., 1999) and neurotransmitters such as GABA and glutamate (Peng et al., 1993). Lastly, the focus of this project, which is the ability of glutamine to increase concentrations of the potent antioxidant glutathione (GSH) (Hong et al., 1992; Harward et al., 1994; Denno et al., 1996; Flaring et al., 2003). The a decrease in GSH concentration has been associated with loss of integrity in the blood brain barrier (Agarwal and Shukla, 1999) and increased signs of lipid peroxidation and pro-apoptotic factors (Genovese et al., 2007). In contrast, increasing GSH after SCI is associated with improved recovery including spinal tissue and motor function (Kamencic et al., 2001).

Improving the maintenance of GSH following injury can be done through manipulation of the GSH synthesis pathway. GSH is a tripeptide that is synthesized in two steps (Meister, 1974, 1988). In step one, the enzyme γ-glutamyl-cysteine ligase (GCL) forms γ-glutamyl-cysteine from glutamate and cysteine. The second step is catalyzed by GSH synthase where glycine is ligated to γ-glutamyl-cysteine. Step one, which provides the substrate for step two, is rate-limiting in several ways including the availability of cysteine and GSH-mediated negative feedback loop affecting (GCL) (Meister, 1974, 1988). It is by manipulating cysteine concentrations and inhibiting this feedback loop that the rate of GSH synthesis can be increased.

Most of the extracellular cysteine is oxidized to cystine: in the CNS cystine is taken up into cells (mainly astrocytes) by the cystine/glutamate antiporter (McBean and Flynn, 2001). As intracellular concentrations of glutamate increase so will the activity of the antiporter and consequently intracellular concentrations of cystine which can then be reduced to cysteine thereby promoting GSH synthesis. GSH, itself, has a feedback inhibition of GCL that is alleviated by glutamate (Meister, 1988); hence, an increase in intracellular glutamate concentration should also increase GSH synthesis by this second mechanism. In principle, one should be able to increase intracellular glutamate by administering glutamine since intracellular glutamine is acted upon by glutaminase within the neuron to give rise to glutamate and ammonia (Kvamme et al., 2001).

Although this project focuses on the improved maintenance of intracellular GSH as the therapeutic mechanism, we must recognize and briefly address the additional routes through which glutamine may function. In states of physiological stress, if the acid/base balance is lost, metabolic acidosis can result. One of the body's responses to acidosis is to increase ammoniagenesis and gluconeogenesis from plasma glutamine.

Glutamine plays a key role in the ammoniagenesis as the most important NH_3 donor (Matthews and Anderson, 2002). Renal glutamine is acted upon by a pH sensitive glutaminase giving rise to NH_3 and glutamate (Gstraunthaler et al., 2000). The NH_3 is then exported to the lumen of the kidney where it binds H^+ to form NH_4 which is excreted in the urine. The binding and excretion of H^+ is essential to the maintenance of blood pH. Renal gluconeogenesis predominantly uses glutamine as a substrate, unlike the liver which uses alanine (Stumvoll et al., 1998). Under normal conditions renal gluconeogenesis contributes up to 25% of circulating plasma glucose (Stumvoll et al., 1999) which can be increased to 50% during metabolic acidosis (Owen et al., 1969). In addition to providing glucose as an energy source glutamine supplementation has also been demonstrated to increase adenosine triphosphate (ATP) production in situations of physiological stress (Khogali et al., 1998; Kumar and Anandan, 2007; Yang et al., 2007).

1.3.3 Concerns over use

Concern surrounding the use of glutamine for any type of neurotrauma is due to the possible increase in extracellular concentrations of glutamate, a potentially excitotoxic neurotransmitter. Curiously, it is by using the cell's ability to increase the concentration of intracellular glutamate that both antioxidants and ATP are produced. Clinically, concentrations of glutamate have been measured in the cerebral spinal fluid (CSF) of neurotrauma patients receiving glutamine and no increases in glutamate have been found (Ronne Engstrom et al., 2005; Berg et al., 2006). Glutamine, in combination with a probiotic has also been used in brain injury patients with findings of decreased

infection rate and length of stay in intensive care (Falcao de Arruda and de Aguilar-Nascimento, 2004).

The objective of this project is to promote GSH maintenance following SCI through increasing intracellular glutamate. In an attempt to increase intracellular cysteine, extracellular glutamate will increase as a result of the increased activity of the antiporter. However, as stated above, if glutamine is given in moderate doses CSF concentrations of glutamate do not change and therefore care must be taken to balance the beneficial and possible detrimental effects of glutamine supplementation.

1.4 Potential of glutamine as a neuroprotectant and hypotheses

Glutamine exerts a positive effect on many tissues regardless of the expansive range of paradigms with an apparent lack of negative side effects. Glutamine has been demonstrated to act as an antioxidant precursor, as previously described in Section 1.3.3. Therefore our first hypothesis was that the administration of glutamine would increase GSH both in cultures astrocytes and *in vivo* following SCI. If our findings supported that hypothesis and glutamine treatment did result in increased GSH we would preceed with testing our second hypothesis.

If glutamine administration increased GSH content after SCI it would decrease spinal cord and body wide oxidative stress which should be measurable in functional recovery. Therefore our second hypothesis was that increasing GSH content will result in more tissue sparing and better locomotor outcome following SCI.

Lastly, if the previous hypothesis is supported by our findings and because of the multifaceted effects of glutamine the effects of glutamine administration after SCI

should be robust to variations in injury. Our final hypothesis is that glutamine treatment efficacy will be reproducible across the two spinal injury models tested.

1.5 Aims

1. To determine the effect of glutamine on the maintenance of GSH in the following paradigms:
    a. Cultured astrocytes (Chapter 2)
    b. In blood and spinal tissues following SCI induced by a modified aneurysm clip (Chapter 2)
    c. Following SCI induced by a modified forceps (Chapter 3 & 4)
2. To determine the effect of glutamine treatment on tissues sparing at the site of injury using both aneurysm clip and forceps SCI models (Chapters 2-4).
3. To determine the effect of glutamine administration following SCI on functional recovery, including micturation and locomotory recovery (Chapters 2-4).

CHAPTER 2.0

EVALUATION OF THE POTENCY OF GLUTAMINE TREATMENT FOLLOWING SPINAL CORD INJURY USING A MODIFIED ANEURISM CLIP

2.1 Introduction

SCI occurs in two phases: the immediate or primary and the secondary. The primary phase, which is thought to comprise only 10% of the overall injury to the tissue (Young et al., 1982), involves the initial mechanical damage to the spinal cord by contusion, compression, laceration and/or shearing. The secondary phase that immediately follows can last for days, weeks or months and is a highly complex process involving numerous inter-related mechanisms including: ischemia, oxidative stress, inflammation, excitotoxicity and apoptosis (Juurlink and Paterson, 1998) with inflammation driving many of the tissue destructive mechanisms (Jones et al., 2005).

The focus of our laboratory has been to decrease oxidative stress following SCI as this mechanism activates signaling pathways such nuclear factor kappa B (NFκB) (Christman et al., 1998), thereby activating inflammatory pathways. Since GSH plays critical roles in many oxidant scavenging pathways (Juurlink, 1999), this project has concentrated on maintaining spinal cord tissue GSH levels following injury. GSH levels decrease markedly in injured spinal cord, even many segments away from the original injury site (Kamencic et al., 2001). One approach we have used to maintain GSH levels in injured spinal cord is to administer the procysteine compound L-2-oxothizolidine-4-carboxylate (OTZ) after a spinal cord crush injury. The rationale behind this approach is that cysteine is the rate-limiting amino acid in GSH synthesis, and OTZ is converted to cysteine intracellularly (Williamson et al., 1982). We have shown that OTZ promotes

maintenance of spinal cord GSH levels thereby decreasing secondary damage allowing better functional outcomes and spinal tissue preservation (Kamencic et al., 2001).

GSH is a tripeptide that is synthesized in two steps (Figure 2-1) (Meister, 1988). In step one the enzyme GCL forms γ-glutamyl-cysteine from glutamate and cysteine. The second step is catalyzed by GSH synthase where glycine is ligated to γ-glutamyl-cysteine. Step one which provides the substrate for step two is rate-limiting in several ways including the availability of cysteine and GSH-mediated negative feedback loop affecting GCL (Meister, 1988). Although cysteine is the rate-limiting amino acid, intracellular glutamate concentrations can affect the synthesis of γ-glutamyl-cysteine in two ways.

Most of the extracellular cysteine is oxidized to cystine: in the CNS cystine is taken up into cells (mainly astrocytes) by the cystine/glutamate antiporter (McBean and Flynn, 2001). As intracellular concentrations of glutamate increase so will the activity of the antiporter and consequently intracellular concentrations of cystine which can then be reduced to cysteine thereby promoting GSH synthesis. GSH, itself, has a feedback inhibition of GCL that is alleviated by glutamate (Meister, 1988); hence, an increase in intracellular glutamate should also increase GSH synthesis by this second mechanism. In principle, one should be able to increase intracellular glutamate by administering the amino acid glutamine since intracellular glutamine is acted upon by glutaminase to give rise to glutamate and ammonia (Kvamme et al., 2001).

Glutamine is the most abundant free amino acid in the body and is classically considered a non-essential amino acid. However, recently many studies are demonstrating the beneficial effects of glutamine supplementation in situations of

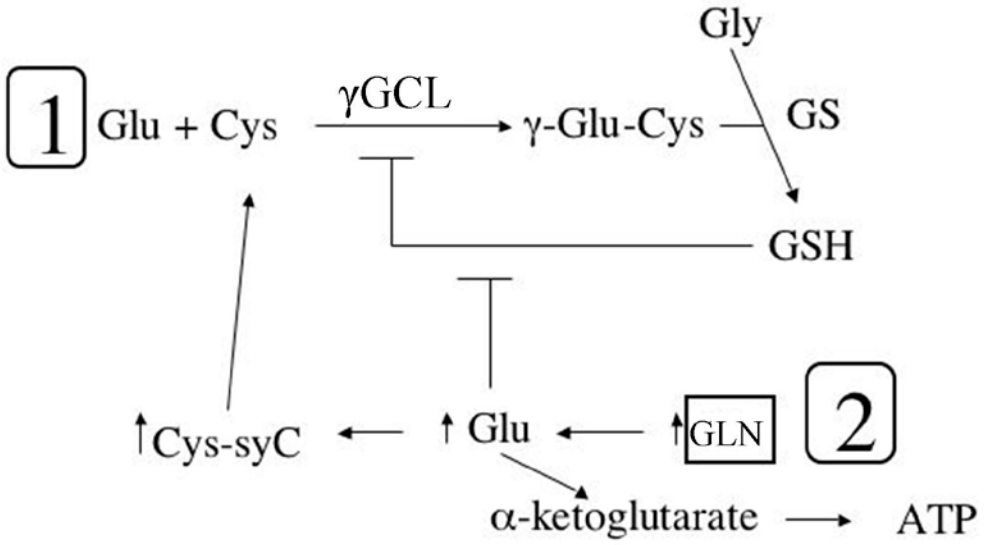


Figure 2-1: The regulation of glutathione (GSH) synthesis. Equation 1 running from left to right demonstrating that the first step in GSH synthesis is the ligation of glutamate (Glu) to cysteine (Cys) to form the dipeptide gamma-glutamyl-cysteine (γ-Glu-Cys) catalyzed by the enzyme γ-glutamyl-cysteine ligase (γGCL). Cysteine is the rate-limiting amino acid in this first step of synthesis. The second step of synthesis is the ligation of glycine (Gly) to γ-Glu-Cys to form GSH. GSH has feedback inhibition on γGCL activity that limits cellular GSH concentrations. Equation 2 running from right to left outlines the therapeutic possibility of glutamine (GLN). Glutamine is converted to glutamate by the enzyme glutaminase. Glutamate influences GSH synthesis in two manners. Firstly, in alleviates the inhibition of γGCL activity by GSH and secondly, it promotes the uptake of cystine (Cys-syC) by the glutamate-cystine antiporter. Cystine is reduced to cysteine, the rate-limiting amino acid in GSH synthesis. In addition, glutamate con be converted to α-ketoglutarate promoting the production of adenosine triphosphate (ATP) in compromised cells.

physiological stress including: gastrointestinal surgery or trauma (Wilmore, 2001), lethal hepatic injury (Hong et al., 1992), brain injury (Falcao de Arruda and de Aguilar-Nascimento, 2004) and in adult burn victims (Garrel et al., 2003).

Due to the overwhelming and continually growing body of data supporting the use of glutamine clinically, this amino acid is now generally accepted as a "conditionally essential" nutrient (Lacey and Wilmore, 1990; Wischmeyer, 2008). The hypothesis underlying this study is that glutamine administration following SCI should promote maintenance of spinal cord GSH levels. Although not examined in this study, glutamine may also work through additional pathways including improving the maintenance of the citric acid cycle function since glutamate can be converted to α-ketoglutarate and, hence, better maintain ATP levels in the injured tissue (Figure 2-1).

The objectives of the experiments described in this manuscript were: 1) to determine whether glutamine administration will increase or maintain cellular GSH levels in a cell culture model and in a rat SCI model, and 2) whether this improved maintenance of GSH concentrations following SCI is correlated with less tissue damage and improved functional outcomes.

2.2 Materials and Methods

2.2.1 Cell Culture Procedures

Rat type I astrocytes were cultured as previously described (Juurlink et al., 1998). Briefly, Wistar rat newborns were killed using an overdose of the anesthetic Methophane, approved by Canadian Council on Animal Care. Neopallial tissues were mechanically disassociated into single cells and planted at a low cell density ($3 \text{ X } 10^5$). Growth medium (DMEM) was supplemented with 7.5 mM glucose, 15 mM $NaHCO_3$, 2

mM glutamine and 10 % (v/v) horse serum and placed in an incubator at 37 °C with a humidified atmosphere comprised of 5 % CO_2 with the balance being air. Cultures were fed three times per week. Confluent cultures were used at the beginning of the third week.

2.2.2 Experimental Animals and Surgical Procedures

Male Wistar rats weighing 250 g at time of surgery, purchased from Charles River Laboratories (Laval, PQ, Canada), were used for all experiments. All protocols used followed the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Council (protocol number 20020112). Rats were housed individually, received standard rat chow and water *ad libitum* and were kept in a 12 hour light/dark cycle at 25 ºC with controlled humidity. Animals were randomly assigned to experimental groups.

The spinal cord compression injury protocol was the same as that used previously in our laboratory (Kamencic et al., 2001; Schultke et al., 2003) and originally described (Rivlin and Tator, 1977). Briefly, in preparation for surgery each rat was anesthetized with 5 % halothane and maintained at 1.5 % halothane for the duration of the surgery (MTC Pharmaceuticals, Cambridge, ON) and a balance of medical grade oxygen (Praxair, Saskatoon, SK). The dorsal mid-thoracic surface was shaved and cleaned with chlorohexidine and 70 % alcohol and the animal is given a pre-surgical injection of buprenorphrine (0.05 mg/kg). A laminectomy was performed on the T6 and T7 vertebrae and an extradural 5 second spinal cord compression on the T6 segment was performed using a modified aneurism clip which gave a closure force of 30 g (Kerr-Lougheed clip, Walsh Manufacturing, Oakville, Ontario). Sham surgeries were

comprised of the laminectomy but without the spinal compression. Manual bladder expressions were performed on all injured animals every 12 hours until the ability to micturate returned or the animal was sacrificed.

2.2.3 Animal Perfusion and Tissue Collection

All rats were sacrificed by trans-cardiac perfusion while anesthetized with 5 % halothane. Animals whose tissues were collected for biochemical analysis were rapidly perfused with cold saline. Exposed tissues were cooled with liquid nitrogen and segments of interested were removed, frozen immediately in liquid nitrogen and stored at -80 ˚C until analyzed. Rats whose tissues were to be used for histology were perfused with 250 mL of phosphate buffered (0.03 M) saline followed by 250 mL of 4 % paraformaldehyde (PFA). The complete spinal column was resected and placed in 4% PFA overnight and then in 10 % phosphate buffered formalin until paraffin embedded.

2.2.4 Measurements of GSH

2.2.4.1 Astrocyte cultures

To measure GSH, monochlorobimane (MCB) was added to cell culture to a final concentration of 100 μM. Thirty minutes after MCB addition, astrocytes were washed twice with phosphate buffered saline. Cells were sonicated on ice (three times for 5 seconds each) with one minute intervals. After centrifugation at 15000 g for 10 min at 4 °C, the supernatant was used to determine GSH content by the MCB method (Kamencic et al., 2000). Total cellular protein was calculated using the bicinchoninic acid method. A 1-way analysis of variance (ANOVA) with post hoc Tukey's test was used to analyze the data. Data are expressed as nmoles GSH/mg protein ± standard error of the mean.

2.2.4.2 Blood samples from injured animals

Blood GSH measurements were performed similar to that previously described (Asensi et al., 1999). Briefly the acidic supernatant was added to a solution containing 0.47 M potassium phosphate, 0.1 M 1-chloro-2,4-dinitrobenzene and 5 units of GSH-S-transferase. Changes in absorbance over five minutes were read at 340 nm in triplicate using a Spectra Max 190 (Molecular Devices) microtitre plate reader and results are represented as average ± standard error. Statistical significance was tested by 1-way ANOVA with a post hoc Tukey's test.

2.2.4.3 Injured rat spinal cord

GSH concentrations in the spinal cord were measured by high-performance liquid chromatography (HPLC) using 5,59-dithio-bis(2-nitrobenzoic) acid-derivatization (DTNB) and ultraviolet (UV) detection (Katrusiak et al., 2001). Briefly, tissues were homogenized in 5 % sulfosalicylic acid containing 0.2 mM ethylenediaminetetracetic acid (EDTA) and sonicated with intermittent cooling. The homogenates were then centrifuged at 10,000 g for 15 minutes and supernatants were collected, derivatized and triplicate samples were analyzed using a Shimadzu reversed-phase HPLC with UV detection. Data were collected digitally with Shimadzu Ezchrom Version 3.2 chromatography software. GSH content in rat spinal segments was expressed in µmol/g wet weight. Data are represented as average ± standard error and were analyzed using a 1-way ANOVA with a post-hoc Tukey's test.

2.2.5 Assessment of motor function

For a minimum of 3 days before surgery all animals were handled twice daily and exposed to the testing surface used for the Basso-Beattie-Bresnahan (BBB) open field locomotor rating scale (Basso et al., 1995). Animals were weighed and BBB scores were measured weekly for 6 weeks by blinded examiners. The examiners scores were averaged and the scores are displayed ± standard error. Data were analyzed by a 1-way ANOVA followed by a post-hoc Tukey's test as recommended (Scheff et al., 2002).

2.2.6 Histology

Spinal segments were isolated using the spinal roots as landmarks for all animals in the experiment (N = 8). Paraffin embedded tissues were sectioned at a 10 µm thickness and every 4th and 5th sections were collected over a distance of 5.0 mm. Sections were then stained with luxol fast blue (LFB) and cresyl violet, as previously described (Kamencic et al., 2001). Slides were assessed visually to narrow down slides containing the epicenter of the injury. Images were collected using a Leica DRMD microscope with a Nikon Cool Pix digital camera of five slides which were then analyzed for the amount of LFB positive tissue. The section containing the least amount of LFB stained tissue was considered the epicenter of the injury.

2.2.7 Quantification of White Matter

White matter was quantified using the Image J software (NIH) with a color deconvolution plugin (Ruifrok and Johnston, 2001). The color deconvolution plug-in was run using the Fastblue, FastRed and DAB program, the background subtracted and the image brought to threshold. The quantification tool was then used to calculate the

number of stained pixels. The analysis was run in duplicate by a blinded examiner and the average of the two measurements was used as the value for the sample. The average number of pixels for the total cross sectional area of the T6 sections from eight healthy animals was measured. Calculations of white matter sparing were performed by dividing the number of LFB positive pixels by the number of pixels in total cross sectional area of the healthy control. Results are displayed as the average percentage ± standard error. Data were analyzed by a 1-way ANOVA with a posthoc Tukey's test.

2.3 Experimental Design

2.3.1 Effect of glutamine administration on GSH content

2.3.1.1 Astrocyte cultures

The cultures were supplied glutamine-free medium for three days. Three culture plates were randomly assigned to each experimental group with 3 separate culture batches being examined. Glutamine was added to the cell culture at the following concentrations: 0 mM, 0.1 mM, 0.5 mM, 1.0 mM and 5.0 mM and incubated for 24 hours prior to harvesting.

2.3.1.2 Blood samples from injured animals

Sixteen animals randomly assigned to either saline or 1 mmol/kg glutamine treatment (N = 8) were used to examine blood GSH content in spinal injured animals. Blood samples were collected from the great saphenous veins using a 25 gauge needle and a 75 mm heparinized hematocrit capillary tube during surgical preparation (-1), 1 hour following surgery (injection time = time 0) and 1, 2, 6 and 24 hours thereafter. The blood was treated with an equal volume of 30 % trichloroacetic acid with 2 mM EDTA

and centrifuged at 15,000 g for 5 minutes. The supernatant was removed and frozen in liquid nitrogen and stored at -80 ˚C until analyzed. All animals received only a single IP injection of 1 mmol/kg glutamine or vehicle control (saline) one hour after injury and were sacrificed 24 hours after injection.

2.3.1.3 Injured rat spinal cord

To determine if glutamine treatment would affect the level of GSH in the spinal cord at the site of injury, 54 animals were randomly assigned into 9 experimental groups (N = 6). Experimental groups include, control (uninjured) group, two sham groups (saline and 5 mmol glutamine/kg body weight) and six groups of injured animals treated with saline or one of the following concentrations of glutamine (10, 5, 2.5, 1.0 and 0.5 mmol glutamine/kg). Animals received IP injections of saline or glutamine 1 hour and 13 hours post-surgery and were sacrificed 24 hours post-surgery. Healthy uninjured animals were assigned an arbitrary time as a time 0 to determine the schedule for injections and sacrifice.

2.3.2 Long term measurements

2.3.2.1 Assessment of motor function

To assess behavioral outcomes and histological changes following injury in the presence or absence of treatment, 16 animals were randomly assigned to either saline or glutamine (1 mmol/kg) experimental groups (N = 8). Treatments were given by IP injection one hour post-surgery and every 12 hours for 7 days and behavioral testing was carried out weekly as described previously. Animals were sacrificed 6 weeks after surgery and spinal cord tissues were collected for histological analyses.

2.4 Results

2.4.1 Effect of glutamine administration on GSH content

2.4.1.1 Astrocyte cultures

Astrocytes cultured for three days in the absence of glutamine had a GSH content of 14.4 ± 1.0 nmoles GSH/mg protein (Figure 2-2), this was significantly increased to 18.6 ± 1.0 when cultured in the presence of 1 mM glutamine ($P < 0.05$) and 24.7 ± 0.7 nmoles GSH/mg protein when cultured in the presence of 5 mM glutamine ($P < 0.001$).

2.4.1.2 Blood samples from injured animals

Within 2 hours of injection (3 hours post-injury) in saline treated animals blood GSH concentrations significantly decreased compared to pre-surgical values (0.545 mmol/L ± 0.005 vs 0.596 mmol/L ± 0.004; $P < 0.05$) and did not recover within 24 hours (0.549 mmol/L ± 0.008 vs 0.596 ± 0.004; $P < 0.05$) (Figure 2-3). Blood GSH values for glutamine treated animals did not significantly decrease ($P = 0.09$) compared to pre-surgical samples; indeed, there was a slight but non-significant increase to 0.627 ± 0.021 mmol/L ($P = 0.07$). Compared to pre-surgical values, animals treated with a single bolus injection of glutamine had significantly better maintenance of blood GSH in samples taken 2, 6 and 24 hours after treatment (Figure 2-3).

2.4.1.3 Injured rat spinal cord

The average GSH content in T6 spinal cord segment in healthy animals was 1.07 ± 0.01 µmol/g tissue (Table 2-1) whereas other segments ranged from 1.01 ± 0.01 to 1.10 ± 0.01 µmol/g tissue. Following surgery in the sham group of animals the GSH

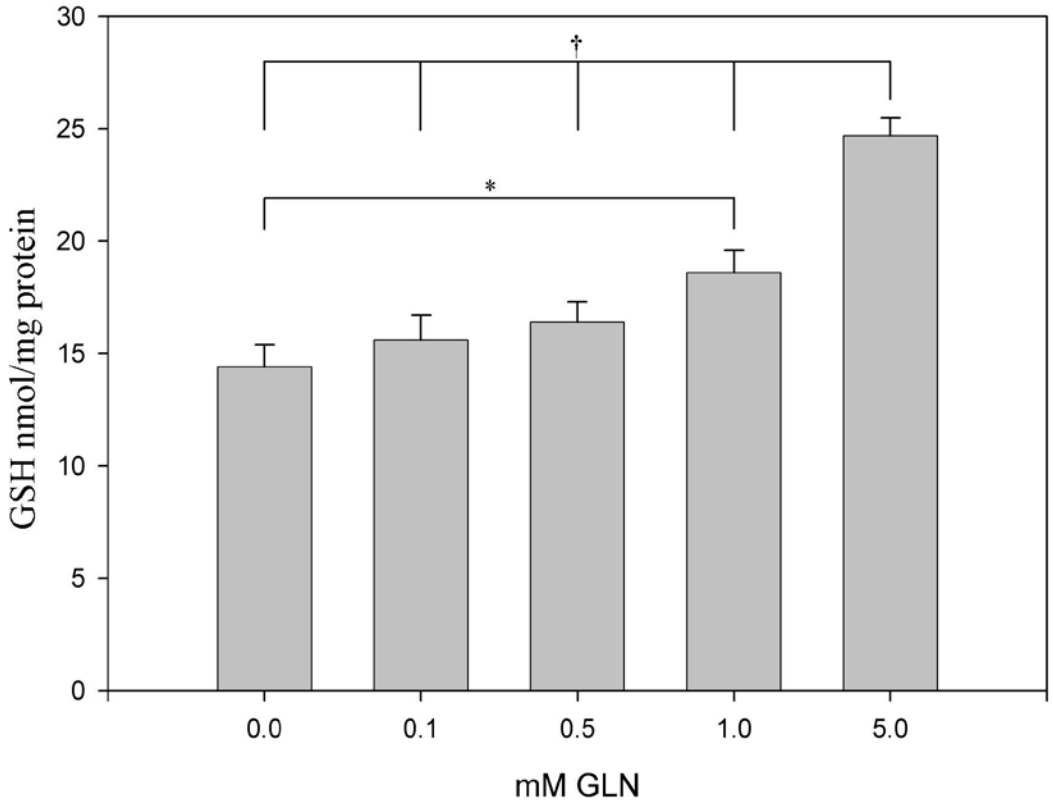


Figure 2-2: The GSH concentration is altered in cultured astrocytes after modulating glutamine content in media for 24 hours. Data are expressed as mean ± standard error. (N = 9) * denotes a significant difference ($P < 0.05$) compared to 1 mM glutamine. † denotes significant differences ($P < 0.05$) between the 5 mM glutamine treatment and all other treatments.

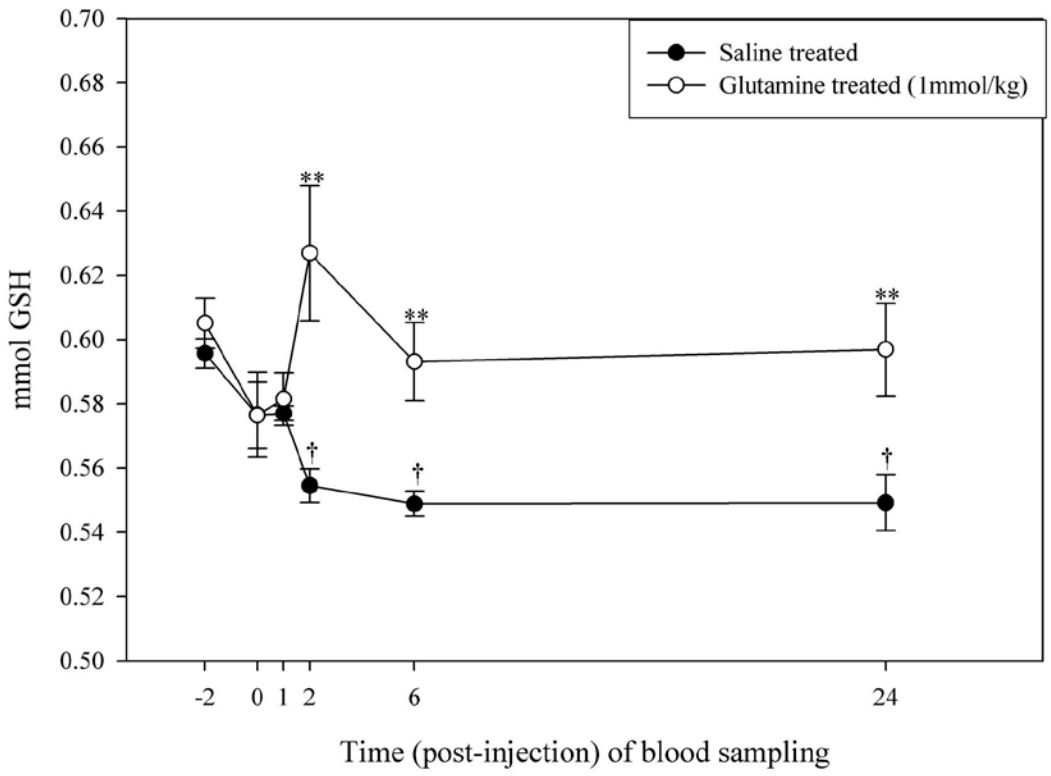


Figure 2-3: Influence of glutamine administration on blood glutathione (GSH) following spinal cord injury. Injury causes a significant reduction in total blood GSH levels within 3 hr after spinal cord injury ($P < 0.05$) and this difference is maintained over 24 hr. A bolus injection of 1 mmol/kg glutamine 1 hour post-injury increases blood GSH concentrations to pre-surgical levels within 2 hours which is maintained for over 12 hours. Data are expressed as mean ± standard error. ($N = 8$) * denotes significant ($P < 0.05$) difference vs saline treated at same time point. † denotes significant difference vs pre-surgical glutamine treated samples.

	C3	T3	T5	T6	T7	T9	L4
Healthy	1.09±0.02	1.01±0.01	1.03±0.01	1.07±0.01	1.10±0.01	1.09±0.02	1.05±0.01
Sham							
Saline	1.05±0.01	1.02±0.00	0.97±0.12	0.92±0.13	1.01±0.08	1.03±0.05	1.02±0.03
5.0 GLN	1.06±0.03	1.02±0.01	0.99±0.10	0.96±0.11	1.02±0.09	1.03±0.07	1.01±0.10
Injured							
Saline	0.92±0.11	0.85±0.07*	0.51±0.06*	0.44±0.05*	0.76±0.09*	0.79±0.11*	0.91±0.11
0.5 GLN	0.94±0.08	0.87±0.12	0.58±0.09*	0.46±0.08*	0.77±0.13*	0.82±0.06*	0.83±0.09*
1.0 GLN	1.01±0.07	0.88±0.05	0.66±0.15*	0.76±0.09†*	0.64±0.09*	0.94±0.07†	0.73±0.05*
2.5 GLN	1.09±0.17	1.03±0.09	0.77±0.16†	0.53±0.13*	0.75±0.23	0.79±0.14	0.90±0.11
5.0 GLN	1.01±0.07	0.83±0.11*	0.75±0.01†*	0.61±0.02*	0.70±0.17*	0.84±0.07	0.81±0.15
10.0 GLN	0.78±0.17*	0.75±0.09*	0.63±0.07*	0.47±0.07*	0.44±0.06†*	0.77±0.04*	0.76±0.03*

Table 2-1: Changes in glutathione concentrations in spinal cord tissues 24 hours after surgery following saline or glutamine (GLN) treatments 1 and 13 hours after surgery. All treatments are expressed as mmol GLN/kg body weight. * denotes a significant ($P < 0.05$) difference compared to healthy controls. † denotes a significant ($P < 0.05$) difference compared to injured saline treated. Data are expressed as µmol/g weight ± standard error.

level at T6, the site of the laminectomy, was slightly but non-significantly decreased. Similarly the sham surgery did not induce GSH changes at the other spinal levels. Administration of 5 mmol/kg glutamine following sham surgery had no effect on spinal cord tissue GSH (Table 2-1).

Following spinal injury, in saline treated animals, GSH levels significantly dropped to 0.44 ± 0.05 µmol/g tissue ($P < 0.01$) at the site of injury (Table 1, Figure 2-4). Similar significant ($P < 0.01$) decreases in GSH levels (Table 2-1) were seen in segments rostral (T3: 0.85 ± 0.07, T5: 0.51 ± 0.06 mmol/g tissue) and caudal to the injury epicenter (T7: 0.76 ± 0.09, T9: 0.79 ± 0.11 µmol/g tissue).

Administration of 1 mmol glutamine /kg body weight 1 hour and 13 hours after injury resulted in a higher concentration of GSH in the spinal cord at the epicenter of the injury 24 hours post-surgery (Table 2-1, Figure 2-4) compared to saline treated (0.76 ± 0.09 vs 0.44 ± 0.05 µmol/g, $P < 0.001$). Administering glutamine at doses of: 0.5, 2.5, 5 and 10 mmol glutamine/kg did not significantly increase T6 GSH concentrations (Table 2-1). Examining GSH levels in segments immediately adjacent to the epicenter of the injury demonstrates that there was a modest improvement in maintenance of spinal cord GSH levels at doses of 0.5 and 2.5 mmol/kg glutamine (Table 2-1). Treatment with 10 mmol/kg glutamine resulted in a lower concentration of GSH compared to the saline treated in the T7 segment, caudal to the epicenter (0.44 ± 0.06 vs 0.76 ± 0.09; $P < 0.01$). Only 1 mmol/kg glutamine resulted in a significantly better maintenance of GSH at the site of primary injury; hence, this dose was chosen for subsequent studies.

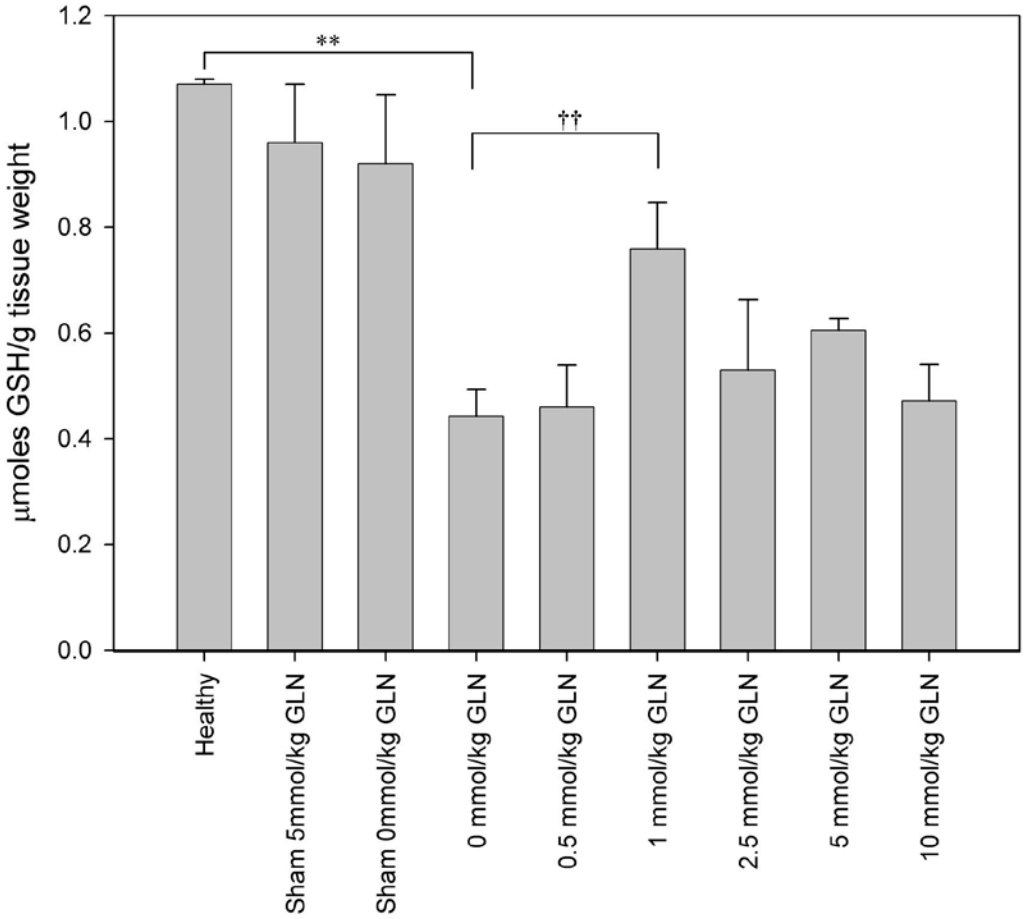


Figure 2-4: Glutamine (GLN) supplementation significantly increases glutathione (GSH) concentrations 24 hours after injury in spinal cord tissue at the site of injury. Data are expressed as mean ± standard error. (N = 6) ** denotes P-value < 0.01 vs healthy control, †† denotes P-value < 0.01 vs saline treated.

2.4.2 Assessment of motor function

Following surgery, all spinal injured animals were paraplegic and all sham (laminectomized only) animals maintained the maximum BBB score of 21. Motor function in glutamine treated animals was significantly better ($P < 0.01$) than the saline-treated animals by 2 weeks following surgery (9.4 ± 1.2 vs 1.7 ± 0.9). This significant difference between treatments was maintained until the 6 week endpoint of the study (9.6 ± 1.3 vs 2.6 ± 0.8) (Figure 2-5).

2.4.3 Histological indicators of damage/recovery

The histological analysis demonstrated that there was a significant loss of spinal cord tissue following injury at the epicenter in tissues collected six weeks following injury (Figure 2-6 A&B). Following SCI, glutamine-treated animals had smaller cystic cavities, increased white matter sparing, better tissue organization and increased tissue area occupied by axons in glutamine treated tissues (Figure 2-6 C&D). Microcystic cavities were seen in all tissues and predominantly in the ventral white matter. In tissues from saline treated animals, spared axons can rarely be found in the white and gray matter whereas in glutamine treated animals, most tissues contain spared axons including intact motor neurons which do not appear in saline treated tissues. The anterior spinal blood vessels are commonly seen intact in both saline and glutamine treated animals. Evidence of immune cell infiltration can also be seen in the presence of macrophages which are seen scattered through the microcysts in the ventral white matter and in the main cystic cavity.

In four, of the eight, glutamine treated animals and only one of the saline treated animals, lamina I and II were spared in the dorsal horn gray matter (Figures 2-6 A&B).

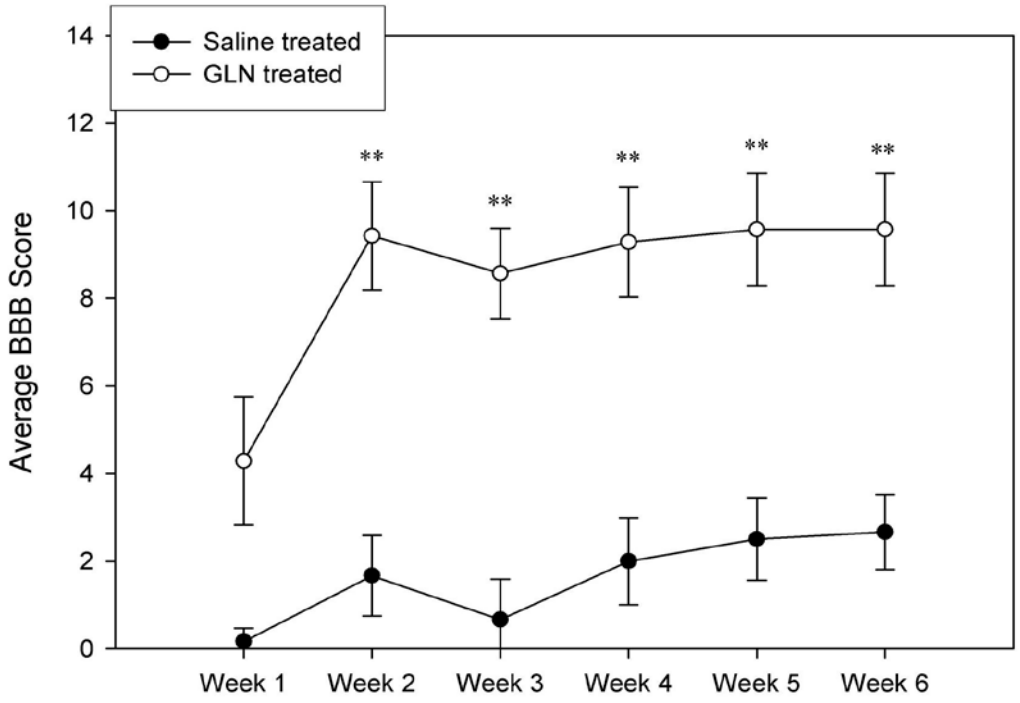


Figure 2-5: Locomotory scores following spinal cord injury. Glutamine supplementation following spinal cord injury significantly increases locomotory recovery as assessed by the Basso Beattie Bresnahan (BBB) scale. Data are expressed as mean ± standard error. (N = 8) ** denotes a significant ($P < 0.01$) difference vs injured saline treated. GLN - glutamine.

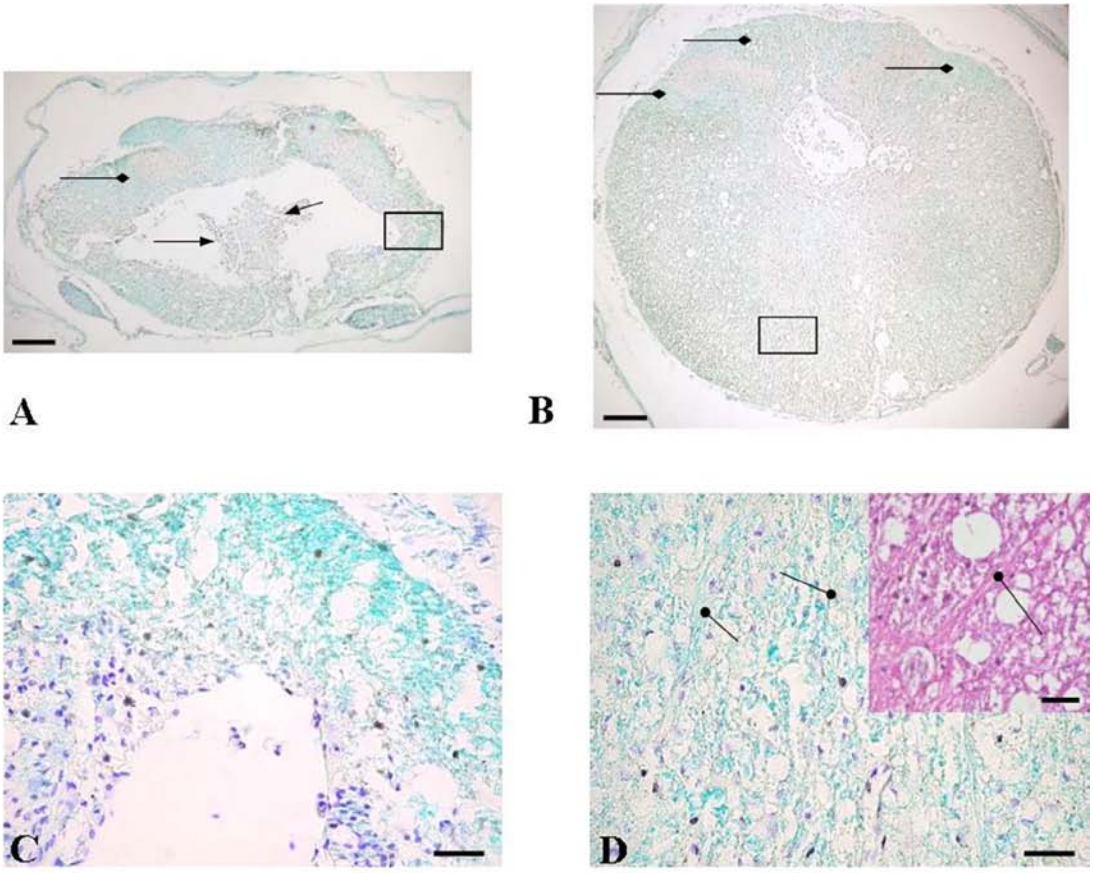


Figure 2-6: Luxol Fast Blue staining demonstrates the significant differences in white matter content between the saline treated (A) and glutamine supplemented (B). Sections are representative images through the injury epicenter. Arrowheads indicate areas of hemorrhagic remnants; diamonds indicate spared white matter while circles indicate axons. Bars = 200 μm (A&B), 50 μm (C&D).

Although these regions are associated with nociception, no indicators of neuropathic pain were observed during daily handling, bladder expressions or injections. At 6 weeks post-injury the saline-treated animals had little or no grey matter remaining and significantly less white matter spared than glutamine treated animals (24.9 ± 5.1% vs 47.1 ± 1.3%, P = 0.003) (Figure 2-7).

2.5 Discussion

2.5.1 Moderate glutamine treatments positively influenced GSH concentrations

Our initial hypothesis that glutamine administration would improve GSH concentrations was supported by our experiments in astrocyte cultures and in blood and spinal tissue samples from spinal injured rats. These findings are likely due to increased intracellular glutamate levels which increase GSH synthesis by the two mechanisms outlined in Figure 2-1. By examining the effect of various doses of glutamine on spinal cord tissue GSH concentrations we were able to determine the optimal glutamine dose. As 1 mmol/kg glutamine was the most potent in maintaining GSH concentrations in whole blood samples, it was chosen as the dose for the long term (6 week) studies.

It is likely that glutamine administration improves outcome following SCI by mechanisms in addition to the promotion of GSH levels. One possible mechanism is improved maintenance of ATP levels in cells adjacent to the primary site of injury. Other mechanisms might include better maintenance of tissues not directly involved in the primary injury. Spinal injured patients have a 46 % decrease in plasma glutamine levels (Rogeri and Rosa, 2005), possibly due to increased utilization and muscle atrophy

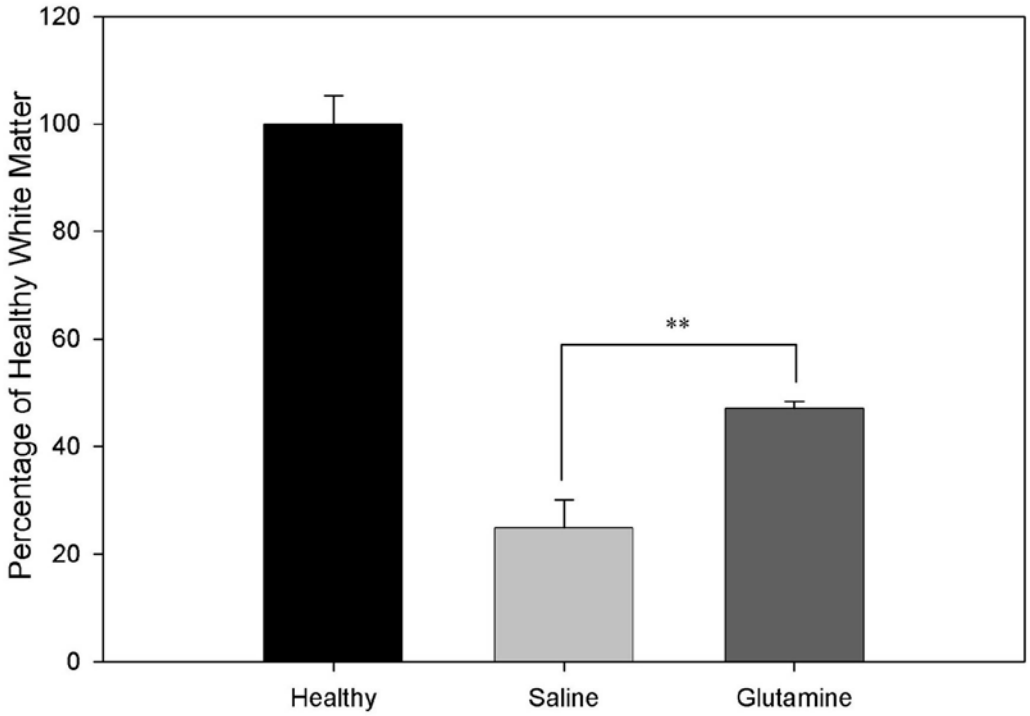


Figure 2-7: Spinal cord white matter content. Spinal cord injury causes a marked decrease ($P < 0.01$) in white matter at the site of injury compared to healthy uninjured. Glutamine treatment following spinal cord injury significantly increases white matter spared at the site of injury. Data are expressed as mean ± standard error. ($N = 8$) ** denotes significant ($P < 0.01$) differences between the saline and glutamine-treated groups.

since muscle is the major source of plasma glutamine. It is known that glutamine is a major energy source for many cells, including immune cells (Oehler and Roth, 2003). This decreased plasma glutamine levels may be responsible for the depressed immune function in spinal injured patients (Rogeri and Rosa, 2005). Glutamine supplementation would be expected to promote the function of not only the immune system but also other body systems.

2.5.2 The question of excitotoxicity

One concern regarding the administration of glutamine in a state of neurotrauma is the possible enhancement of the excitotoxic mechanisms which contribute to tissue damage following SCI (Park et al., 2004). Glutamine administration may result in intracellular glutamate levels rising sufficiently to cause reversal of the cystine/glutamate antiporter or the Na^+-dependent glutamate transporter (Li et al., 1999). Reversal of either or both of these mechanisms may increase extracellular glutamate concentrations to detrimental levels.

Administration of 10 mmol/kg glutamine resulted in a decrease in spinal cord GSH concentrations beyond that of saline treated animals (Table 2-1). This finding suggests that at this concentration there may have been excitotoxic damage due to the glutamine treatment; further experiments are needed to verify this interpretation. In all experiments there were no indicators of excitotoxicity at any concentration below 10 mmol/kg.

In agreement with this interpretation of the relative safety of glutamine administration following neurotrauma is a recent clinical trial involving patients with severe head injuries (Berg et al., 2006). These patients were administered 0.34 g/kg

body weight of the glutamine prodrug L-alanyl- L-glutamine intravenously over a 20-24 hour period. This was similar to the dose to our experiments where we delivered a total of 0.28 g glutamine /kg body weight during a 24 hour period. Using intrathecal microdialysis in the penumbral region adjacent to the site of trauma, Berg et al. (2006) demonstrated that glutamine administration did not increase extracellular glutamate levels. These findings suggest that equivalent glutamine administration in spinal-injured patients will not influence glutamate concentrations in the CSF.

2.5.3 Glutamine treatments improved motor function and sparing of neurologic tissue

We have previously shown that if one could better maintain GSH in injured spinal cord this is associated with less tissue damage and, hence, better locomotory recovery (Kamencic et al., 2001). This improved GSH status following glutamine administration was associated with better spinal cord tissue preservation as well as better locomotory recovery. All of the animals in the glutamine treatment group recovered "extensive movement of all three joints of the hindlimb" (Basso et al., 1995) (BBB score of 7) compared to none in the saline treatment. Four of the 8 animals on glutamine treatment attained a BBB score of 9 or "plantar placement of the paw with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping" (Basso et al., 1995). Not one of the saline-treated animals attained a BBB score of 9.

Histology demonstrated a correlation between white matter sparing and motor function improvement as expected. Similar to previous studies (Joshi and Fehlings, 2002; Kloos et al., 2005), we found microcysts predominantly in the ventral white

matter. In half (4 of 8) of the glutamine treated animals and in one of the eight saline treated animals, lamina I and II of the dorsal horns were spared. This may be a result of a lesser secondary injury due to the glutamine treatment. Tissue destruction following injury emanates from the center of the tissue towards the periphery; therefore, if the injury that develops is of a lesser severity, it may be because laminae I and II are at the periphery that they are spared. Additionally, Joshi and Fehlings (2002) found that the ventral gray matter and columns were more susceptible than the dorsal aspect of the spinal cord.

The correlation seen here between the histological information and functional recovery is difficult to compare with other studies due to the wide variety of methods for calculating tissue sparing and recovery of motor function. There is great variability in outcomes for saline treated animals that underwent the 50 g injury (Fehlings and Tator, 1995; Kamencic et al., 2001; Schultke et al., 2003) which may be due to the decreased reproducibility of the BBB scale in severely injured animals (de Barros Filho and Molina, 2008).

2.5.4 Possible additional mechanisms of action

Given the potency of the therapeutic effect of glutamine, mechanisms other than GSH maintenance should be considered. As glutamine has been demonstrated to improve ATP concentrations following hemorrhagic shock (Yang et al., 2007), this is an additional mechanism through which glutamine may improve tissue sparing at the site of injury. Other mechanisms might include better maintenance of tissues not directly involved in the primary injury.

Spinal injured patients have a decrease in plasma glutamine levels (Rogeri and Rosa, 2005), possibly due to muscle atrophy since muscle is the main source of plasma glutamine. It is known that glutamine is a major energy source for many cells, including immune cells (Oehler and Roth, 2003). The effect of glutamine on immune system functioning after potent physiological stress has been widely studied (Grimble, 2001; Newsholme, 2001; Garrel et al., 2003; Bistrian, 2004; Peng et al., 2006a). Glutamine supplementation had been demonstrated to repeatedly aid in improvement or maintenance of immune function after trauma or surgery.

The decreased plasma glutamine level seen following spinal injury may be responsible for the depressed immune function in spinal injured patients (Rogeri and Rosa, 2005). Glutamine supplementation would be expected to help maintain the function of the immune system which may have therapeutic effects. Although prolonged inflammation within the CNS had been demonstrated to be detrimental (Neumann and Wekerle, 1998), a delayed immune response has been shown to be beneficial and aid in recovery (Schwartz and Yoles, 2006). Stimulated macrophages increased motor function in spinal transected rats to a BBB score of 8 by improving neurite outgrowth across the injury site (Rapalino et al., 1998). It is plausible that in addition to improving ATP and GSH concentrations, that glutamine, by maintaining immune system function would provide yet another mechanism for recovery of function.

2.5.5 Concluding remarks

In this study, we have shown that glutamine supplementation promoted better maintenance of GSH levels in injured spinal cord which was positively correlated with better preservation of tissue and was associated with better functional outcome. The

potency of the treatment, improving BBB scores by more than 6 points, is such that is rarely seen in this SCI model (Kamencic et al., 2001; Schultke et al., 2003; Ackery et al., 2006; Ditor et al., 2007).

Although the currently accepted treatment for SCI is MPO, there is much debate over the use of MPO clinically as currently reviewed (Hurlbert and Moulton, 2002; Hurlbert, 2006; Sayer et al., 2006). The benefit to risk ratio is quite low (Hurlbert, 2000, 2006) and has lead to the questioning of the wide spread use of the compound. As this standard SCI treatment carries with it numerous serious side effects, the low cost, relatively high potency and safety of a treatment such as glutamine becomes even more paramount.

In a number of clinical trials glutamine supplementation has been demonstrated to be clinically relevant in states of physiological stress. To our knowledge, to date there are no studies demonstrating a negative side effect of glutamine supplementation in physiologically relevant concentrations. In contrast, there are an ever growing number of manuscripts detailing the benefits of glutamine supplementation in a wide variety of pathological situations as recently reviewed (Wischmeyer, 2008). Since glutamine supplementation has shown such promise clinically and has had potent therapeutic effects in spinal injured rats, we suggest that human clinical trials should be initiated to determine whether glutamine supplementation would also have therapeutic effects in the acutely spinal injured human.

CHAPTER 3.0

A RELIABLE AND INEXPENSIVE GRADED MODEL OF MODERATE TO SEVERE SPINAL CORD INJURY IN RATS

3.1 Introduction

In order to fully understand the complex mechanisms involved in SCI, researchers must be able to reproduce clinically relevant injuries in the laboratory setting. In humans, most spinal cord injuries occur due to vertebral dislocation or vertebral fracture resulting in contusion and compression of the spinal cord (Tator, 1983; Pickett et al., 2006). SCI can also occur as a result of laceration, transection (Tator, 1983) or hyperflexion and hyperextension of the vertebrae which causes shearing within the spinal cord (Silberstein and McLean, 1994). An attempt has been made in research labs to develop spinal injury models which mimic the damage seen clinically and therefore give researchers a more accurate assessment of the therapeutic potential of interventions.

Current SCI models include methods of contusion, compression, laceration and transection of the spinal cord. Compression and contusion injuries are the most widely used methods for SCI research as an attempt to mimic the neurotrauma seen clinically. Contusion injuries can be induced by the NYU impactor (Gruner, 1992; Basso et al., 1996) or OSU ESCID (Behrmann et al., 1992; Kloos et al., 2005) and the Infinite Horizon SCI device (Scheff et al., 2003) impactor models. Compression injuries have been induced using modified aneurysm clips (Rivlin and Tator, 1978a), epidural balloon (Kobrine et al., 1979; Khan and Griebel, 1983b; Sheng et al., 2004) or modified forceps (Blight, 1991; Gruner et al., 1996).

One important difference between compression and contusion models is the lack or presence of disruption of vascular supply to the spinal cord. Contusion models appear to involve only mechanical primary damage while compression models also affect spinal perfusion (Khan and Griebel, 1983a). Complete or partial transection is also used as an SCI model (Grill et al., 1997; Li et al., 1997; Rapalino et al., 1998) however, as it occurs only rarely clinically (Norenberg et al., 2004), it may not be the optimal model for assessing potential of neuroprotective strategies in SCI. Laceration models of funiculi lesioning and transection can be a useful tool for the study of neurite outgrowth after laceration injury and to further define the role of a spinal cord region. In SCI research the choice of model is a critical one. For these experiments we chose to study a thoracic SCI to model paraplegia. As the majority of thoracic SCI are due to vertebral fractures (Pickett et al., 2006) which result in acute compression of the spinal cord after injury (Tator, 1983), we decided to use a compression injury model.

The Blight modified forceps model (Blight, 1991) (Figure 3-1) was chosen as the SCI model for this project because of its clinical relevance, relatively simple design, ease of use, consistent reproducible injury, availability, low cost and the rapid turnover between surgeries (15-18 animals / day). This model induces an extradural medio-lateral compression of the spinal cord to a defined compression width using a pair of modified coverslip forceps. It was originally described using guinea pigs (Blight, 1991) and then modified for use in rats by Gruner et al. (1996) who described the model using a range of compression widths from 0.8 and 1.8mm. The injury induced was a mild to moderate injury showing classical signs of spinal compression injury both in behavioural and histology outcomes (Gruner et al., 1996).

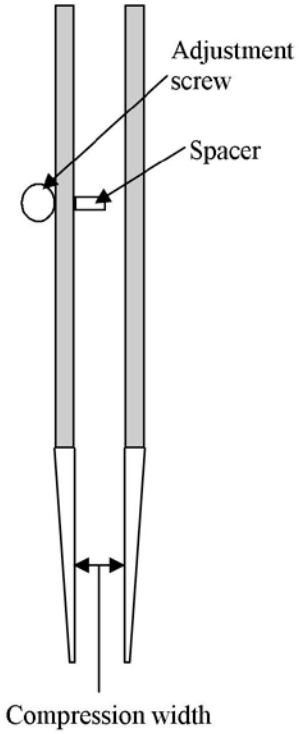


Figure 3-1: Model diagram of the modified German steel forceps used to induce spinal injury. The severity of the spinal injury is determined by the width to which the spinal cord is compressed, referred to as the "compression width". The compression width is altered by the adjusting the screw which lengthens or shortens the spacer between the blades of the forceps.

The goal of this study was to establish a moderate to severe model of SCI by expanding the work of Gruner et al. (1996). With this goal in mind the compression width range of 0.8 - 1.8 mm was expanded down to 0.2 mm and included both the 0.8 mm and 1.0 mm injuries for comparison. The experiment was designed to analyze short term oxidative stress response and long term outcomes measures including return of micturition, locomotory function and histological changes. Our results were then compared to that of previous work using the Blight forceps.

3.2 Materials and methods

3.2.1 Modifying forceps for use in spinal compression injury surgery

German steel coverslip forceps were modified by grinding down the blades to a thickness of 1 mm and a width of 2.5 mm and inserting a screw into the upper portion of the handle (Figure 3-1). The screw could then be adjusted so to alter the “compression width” (width to which the spinal cord would be compressed) which was calibrated using sterilized sparkplug gauges. The forceps were then calibrated prior to each spinal compression and checked immediately after use to ensure accuracy.

3.2.2 Animals

See Section 2.2.2

3.2.3 Surgery and tissue collection

See Section 2.2.2 and 2.2.3 with the exception of the injury protocol which is described below. The spinal cord compression injury induced by modified forceps protocol was similar to that used in Gruner et al. (1996). The forceps were calibrated

using a sparkplug gauge to ensure the accuracy of the compression width and the blades of the modified forceps were placed lateral to the spinal cord at the T6 segment being careful not to damage the spinal roots. To induce the injury, pressure was exerted on the screw and directly opposite to ensure proper closure of the forceps which were closed slowly, over 2 seconds, held for 15 seconds and removed. After removal the compression width was checked again for accuracy.

3.2.4 Blood GSH measurements

Blood collections were performed using the protocol in Section 2.2.4.2 and occurred during the surgical preparation (-1 hour) and 1, 6, 24, 48 and 72 hours after the time of the time of injection (time 0 = 1 hour post surgery).

3.2.5 Evaluation of bladder function

A crude evaluation of micturition was performed which involved the quantification of days post-surgery until the animal's bladder was a small size or the animal did not require manual bladder expression (Engesser-Cesar et al., 2005; Plemel et al., 2008). Evaluation of bladder size and expression of urine, if required, occurred throughout the 6 week span of the study. Bladder sizes were categorized into very small, small, medium, large and very large and were recorded twice daily.

The first indicator of the recovery of micturition was the number of days following the surgery until the first day (of at least 3 consecutive days) the animal's bladder was assessed as being a small size. The second indicator was the number of days following surgery until the first day the animal did not require manual bladder expression (must be the first day prior to recovery of micturition).

3.2.6 Testing of functional performance

See Section 2.2.5

3.2.7 Histology

See Section 2.2.6

3.2.8 Experimental design

To characterize the forceps model in a more severe range of injuries 48 male Wistar rats were randomly divided into 6 experimental groups (N = 8), a sham (laminectomy only) group and one group for each level of severity being examined (0.2 (most severe), 0.4, 0.6, 0.8 and 1.0 (least severe) mm). We chose to include 3 novel injuries (0.2, 0.4 and 0.6 mm) in addition to replicating two that were previously studied (0.8 and 1.0 mm) to determine the reproducibility between laboratories. All animals received injections of saline 1 hour post-surgery and every 12 hours for 1 week. For additional analysis, data from individual injuries was grouped into 2 categories: severe (0.2, 0.4 and 0.6 mm) and moderate (0.8 and 1.0 mm) similar to that seen previously (Gruner et al., 1996; Plemel et al., 2008).

3.2.9 Statistical analysis

With the exception of blood GSH, all data from individual injury groups were analyzed using a 1-way ANOVA and a post-hoc Tukey's test. Blood GSH measurements were assessed for significance using a 2-way ANOVA with post-hoc Tukey's. Differences were considered significant if the P value < 0.05. Pearson's correlation coefficients were calculated for each data set and correlations were considered significant if $P < 0.05$. Correlation coefficients were used to assess the

relationship of each measurement to injury severity in addition to each other. Grouped data were analyzed using a *t* tests. With the exception of the bladder function data, which was as expressed as mean ± standard deviation, all data was expressed as mean ± standard error.

3.3 Results

3.3.1 Blood GSH concentrations following SCI

Blood samples taken from the 0.2 and 0.4 mm spinal injured animals showed decreases in GSH concentrations within an hour of injury; however, only the 0.4 mm injury group reached statistical significance (P = 0.036) and both groups saw a rebound at 6 hours (Table 3-1). Collectively, the lowest concentrations of GSH were seen in the 24 hour blood samples when the three most severe injury groups (0.2 – 0.6 mm) had significantly lower concentrations of GSH than the pre-surgical blood samples (Figure 3-2). Similar to the animals that underwent the sham surgery, the 0.8 and 1.0 mm injury groups had no significant changes in blood GSH at any time point. When the data were grouped, the difference between severe injuries and moderate injuries was more prominent (0.558 ± 0.010 vs 0.584 ± 0.008) and a significant difference (P = 0.024) was seen between injury groups.

3.3.2 Evaluation of bladder function

All animals recovered bladder function within the 6 week time frame of this study and sham animals did not require manual bladder expression. The number of days following surgery until a small bladder size was measured ranged from 7.6 ± 3.1 to 5.6 ± 2.1 for small bladder size and 11.2 ± 2.9 to 9.0 ± 2.2 for recovery in the 0.2 and 1.0

	Pre-surgery	Post-surgery				
		1hr	6hrs	24hrs	48hrs	72hrs
Sham	0.61 ± 0.01	0.60 ± 0.02	0.60 ± 0.01	0.59 ± 0.02	0.61 ± 0.01	0.61 ± 0.01
0.2 mm		0.57 ± 0.03	0.59 ± 0.02	0.56±0.02*	0.59 ± 0.02	0.61 ± 0.02
0.4 mm		0.58±0.01*	0.61 ± 0.03	0.55±0.01*	0.61 ± 0.02	0.59 ± 0.01
0.6 mm		0.61 ± 0.01	0.59 ± 0.01	0.55±0.01*	0.61 ± 0.02	0.59 ± 0.02
0.8 mm		0.61 ± 0.01	0.61 ± 0.02	0.59 ± 0.01	0.59 ± 0.02	0.59 ± 0.01
1.0 mm		0.61 ± 0.02	0.59 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.59 ± 0.02

Table 3-1: Changes in GSH concentrations in whole blood samples taken before and at various times after SCI. Time 0 was one hour after surgery and the time of injection. Significant ($P < 0.05$) differences were found between pre-surgery and post-injury samples are indicated by *. Data are expressed as mmol GSH ± standard error.

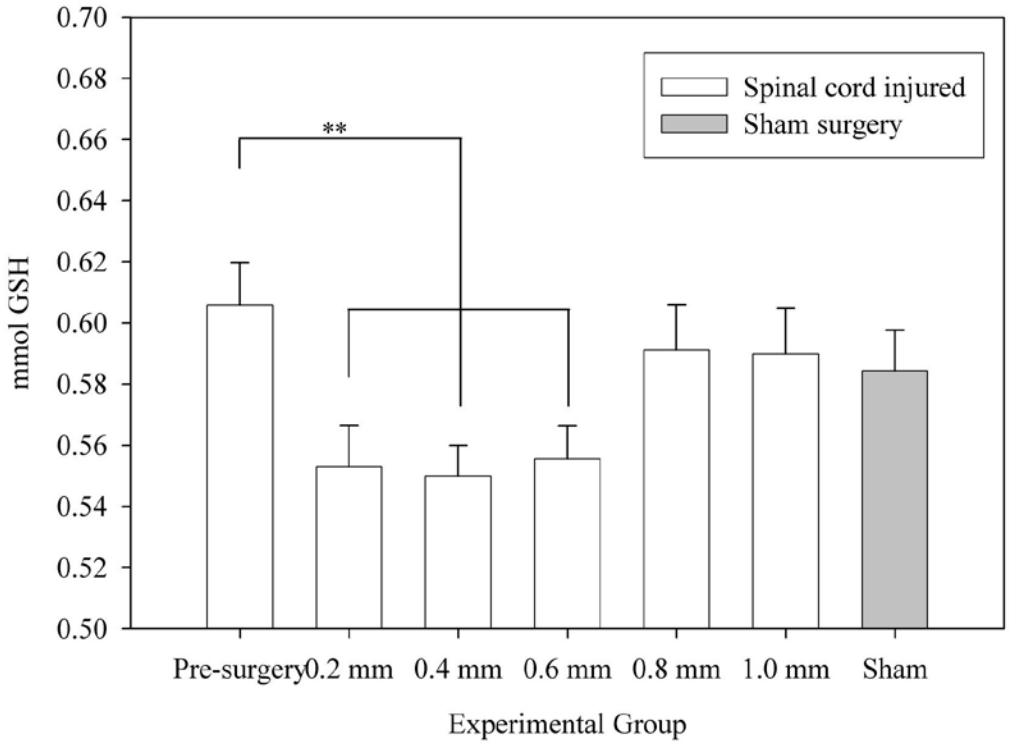


Figure 3-2: Severe spinal cord injury causes a significant decrease in whole blood glutathione concentrations 24 hours after injection (25 hour post surgery) compared to pre-surgery samples. No correlation was seen between injury severity and blood glutathione (GSH) concentrations at any time point. Data are represented as mean ± standard error. ** indicates signficant difference from pre-surgery control, $P < 0.01$.

mm injury respectively (Figure 3-3). No significant differences were found between individual injury groups for either measurement. When injury groups were analyzed, significant differences were found using both the small bladder 8.2 ± 2.1 vs 5.7 ± 2.5 (P = 0.048) and recovery 11.2 ± 2.2 vs 9.1 ± 2.2 (P = 0.014) measurements (Figure 3-4).

3.3.3 Recovery of Motor Function

All sham animals achieved and maintained a BBB score of 21 and an inclined plane score of 55 degrees over the course over the 6 week experiment. Average BBB scores 6 weeks after injury ranged from 7.3 ± 1.1 in the most severe injury (0.2 mm) to 12.2 ± 1.4 in the least severe injury (1.0 mm) (Figure 3-5). The only significant differences were found between the 1.0 mm injury group and the 0.2 (P = 0.001) and 0.4 mm (P = 0.048) injury groups. When grouped, data also showed significant differences between the severe and moderate injury groups (P = 0.031).

One landmark of motor recovery is the “extensive movement of all three joints of the hindlimb” (Basso et al., 1995) which corresponds to a BBB score of 7. Five of the eight 0.2 mm injured animals achieved a 7 or higher while the entire 1.0 mm group achieved it. Another landmark is the BBB score of 9 “plantar placement of the paw with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping” (Basso et al., 1995). In the 0.2 mm injury group, 2 of the 8 animals achieved a 9 or higher on the BBB scale while in the 1.0 mm injury group 4 of the 8 animals achieved a 9 or higher.

The average inclined plane score in the most severely injured animals (0.2 mm) was 32.5 ± 1.9, while animals that received the 1.0 mm injury achieved a substantially higher score of 41.7 ± 2.7 (Figure 3-6). Significant differences were found between the

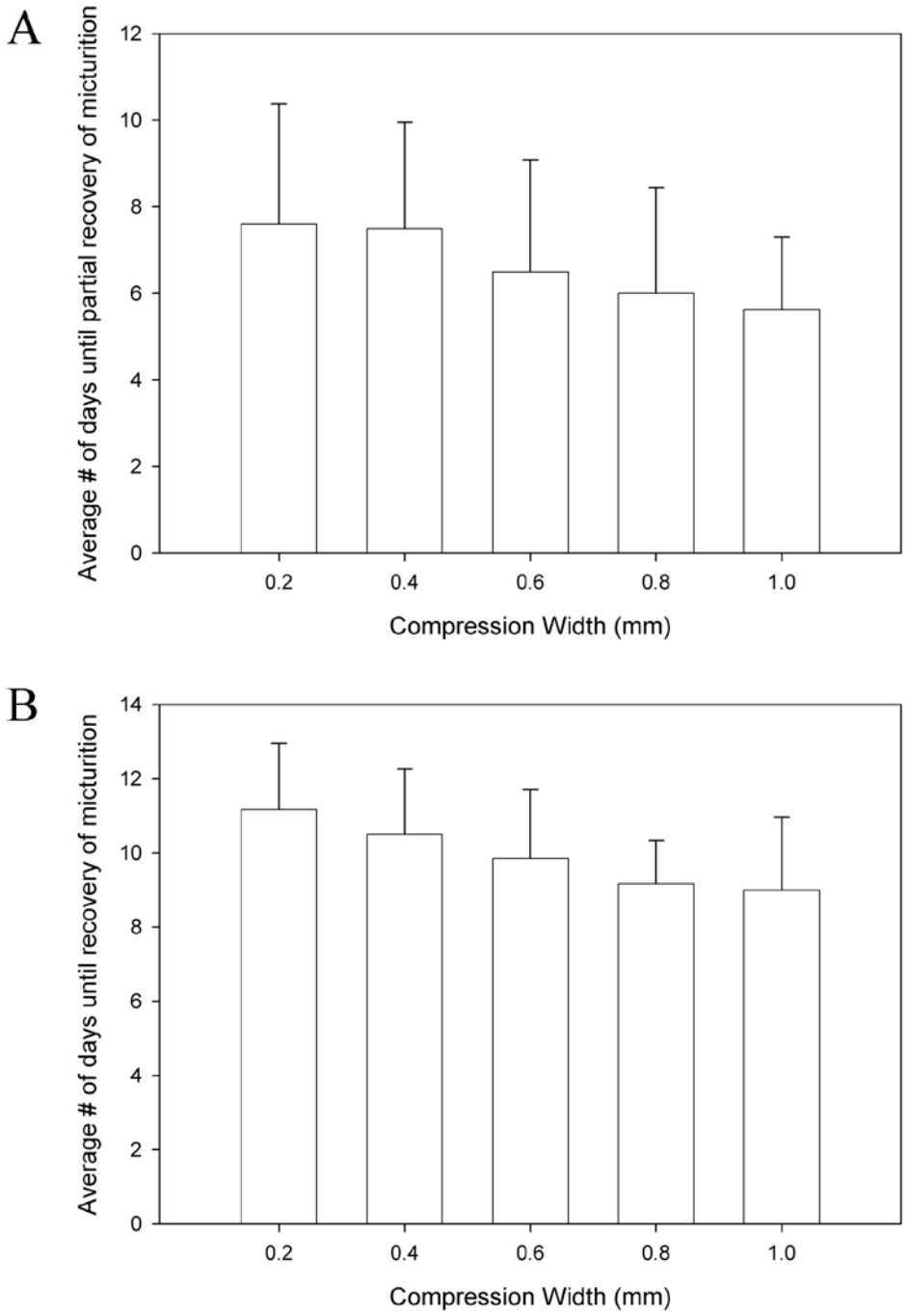


Figure 3-3: Return of micturition following spinal cord injury correlates significantly with the severity of injury. Micturition was assessed by quantifying the days post-surgery until the bladder size was first assessed as small (A) or recovery of micturition (B). Correlation coefficients indicated a negative relationship by either the first assessment of a small bladder size (A: $r^2 = -0.566$) or recovery of micturation (B: $r^2 = -0.585$). No significant differences were detected between individual injury groups. Data are presented as mean ± standard deviation.

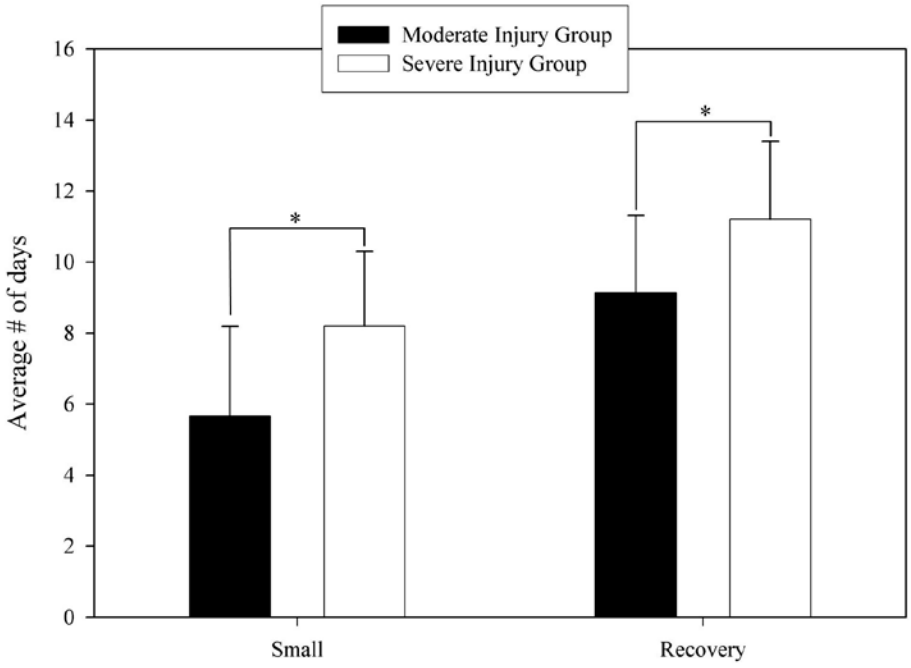


Figure 3-4: Rate of micturition recovery significantly differed for both small bladder and recovery measurements when injuries were grouped as moderate or severe. The moderate injury group included injuries 0.8 - 1.0 mm while the severe group included 0.2 - 0.6 mm injuries. Data are presented as mean ± standard deviation. * denotes significant difference between groups ($P < 0.05$).

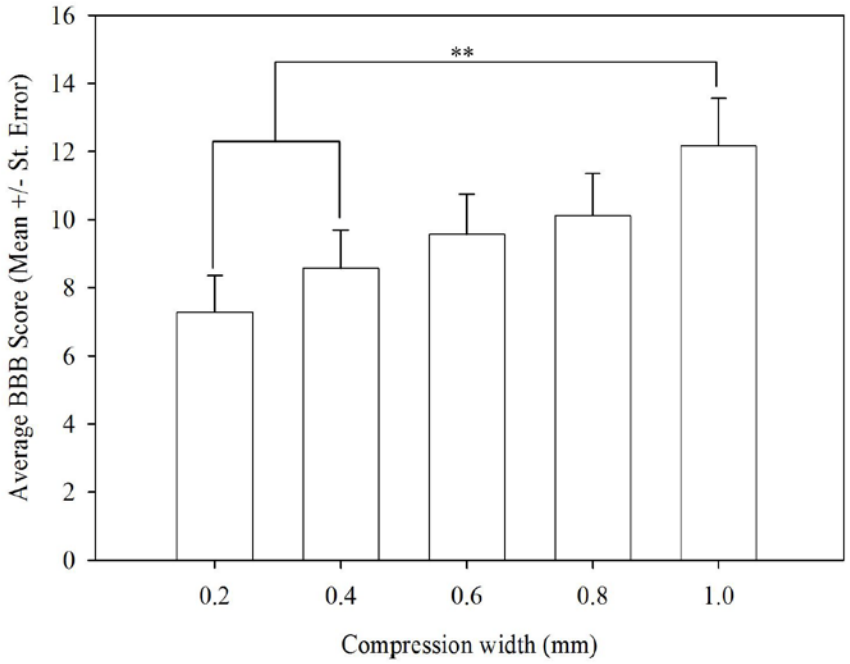


Figure 3-5: Decreased injury severity resulted in higher BBB scores 6 weeks after injury. Rats that underwent the least severe (1.0 mm) injury had significantly higher BBB scores than both the 0.2 and 0.4 mm groups. BBB scores correlated strongly with the severity of the injury ($r^2 = 0.663$, $P < 0.001$). Data are presented as average ± standard error. ** indicates a significant difference ($P < 0.01$) from the 1.0 mm.

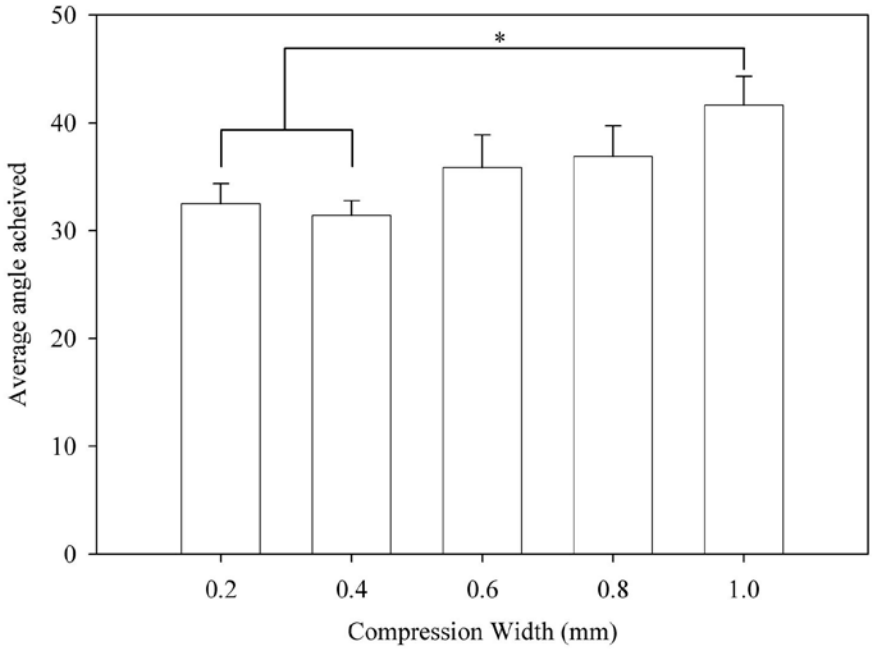


Figure 3-6: Inclined plane scores significantly increase with decreased injury severity. Animal receiving the mildest injury tested had significantly higher inclined plane scores than the two most severe injuries. The angle achieved in inclined plane measurement significantly correlated with injury severity ($r^2 = 0.652$, $P < 0.001$). Data are presented as average angle ± standard error. * denotes a significant different, $P < 0.05$.

1.0 mm injury and both the 0.2 mm (P = 0.011) and 0.4 mm (P = 0.002) injuries. In addition, when injuries were grouped there was a significant difference between the injury groups (Figure 3-7) (P = 0.001).

3.3.4 Histological analysis

No detectable damage was seen to the spinal cords of laminectomy animals (Figure 3-8 A). In animals subjected to SCI, spinal cord sections taken from the injury epicenter demonstrated a significant loss of both white and gray matter (Figure 3-8 B-F). Microcysts, cell infiltrates and large cystic cavities were commonly seen in injured tissues, similar to what has been previously documented for experimental SCI in rats (Behrmann et al., 1992; Basso et al., 1996; Gruner et al., 1996; Kloos et al., 2005). Central and ventral gray matter tissues were lost in all injured samples; however, the dorsal horns were occasionally spared, more often in animal receiving the 0.8 and 1.0 mm injury.

The white matter spared in the most severely injured tissues was seen as a superficial rim with a variable width. In animals from the 0.2 mm injury group, no evidence was seen of maintained white matter in the areas of the rubrospinal (RST) or corticospinal tracts (CST). This was difficult to assess due to the tissue disruption following injury. In the milder injuries (0.6 - 1.0 mm) some sparing was seen in the area of RST but only the 1.0 mm injury group had sparing in the area of the CST (Figure 3-8 D-F). The average percentage of remaining white matter ranged from 4.18 ± 0.28 % in the 0.2 mm injury to 8.49 ± 0.36 % in the 1.0 mm injury (Figure 3-9). Data clustered into 3 groups, 0.2 - 0.4 mm, 0.6 and 0.8 - 1.0 mm injuries each group was different from each other but not within itself.

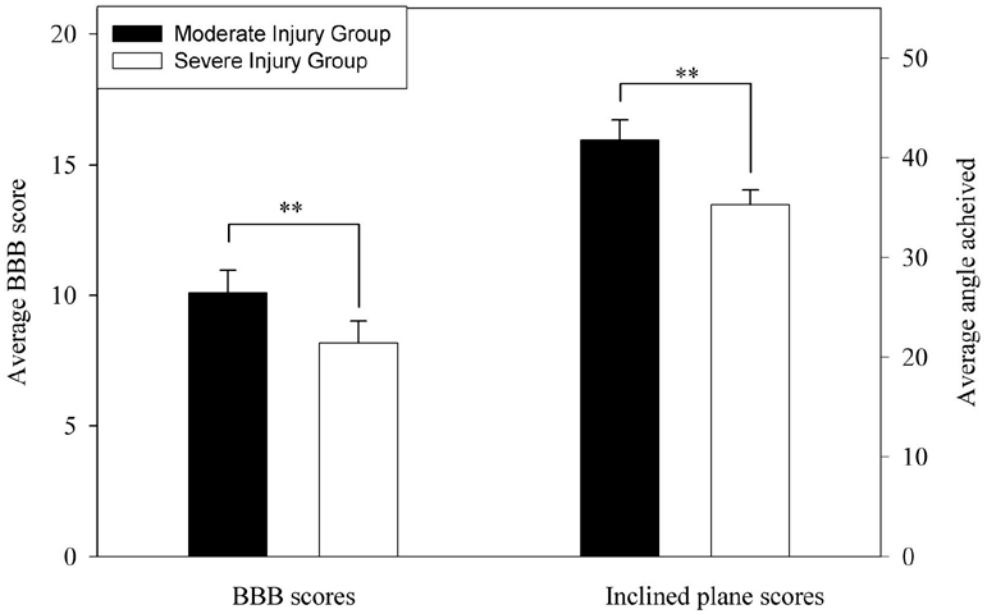


Figure 3-7: Significant differences in both behavioural scores are seen when data is grouped into severe and moderate injury groups. Severe (0.2—0.6 mm) and moderate (0.8—1.0 mm) injury groups significantly differ both in BBB and inclined plane scores. Data are presented as mean ± standard error. ** denotes significance ($P < 0.05$).

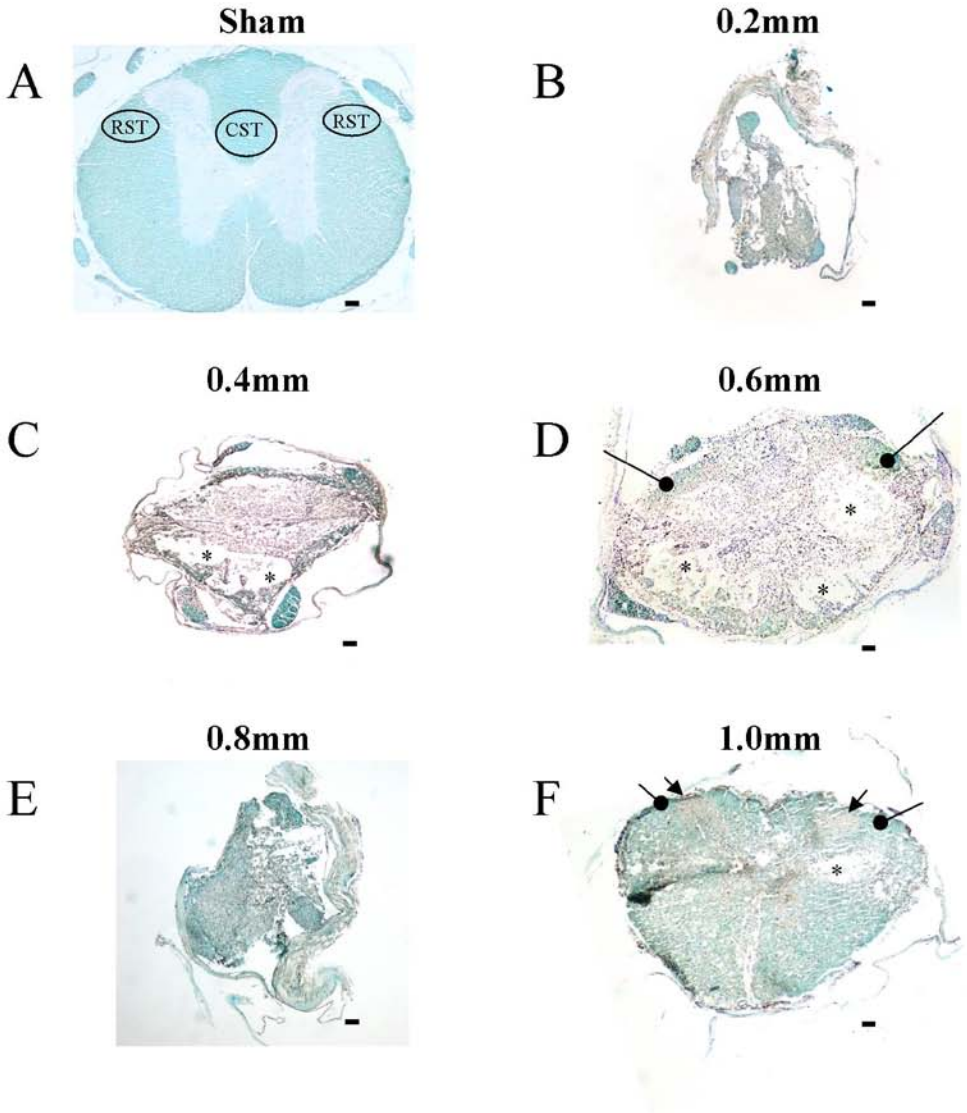


Figure 3-8: Representative histology from injury epicentre in animals receiving laminectomy (A) and spinal compression widths ranging from 0.2 - 1.0 mm (B - F). Increasing injury severity significantly increased tissue loss following SCI. RST - rubrospinal tract, CST - corticospinal tract indicate areas * denotes areas of cystic cavities, black arrows indicate areas of spared white matter, round-ended arrows indicate areas of the RST that are spared, Scale bar represents 100 μm.

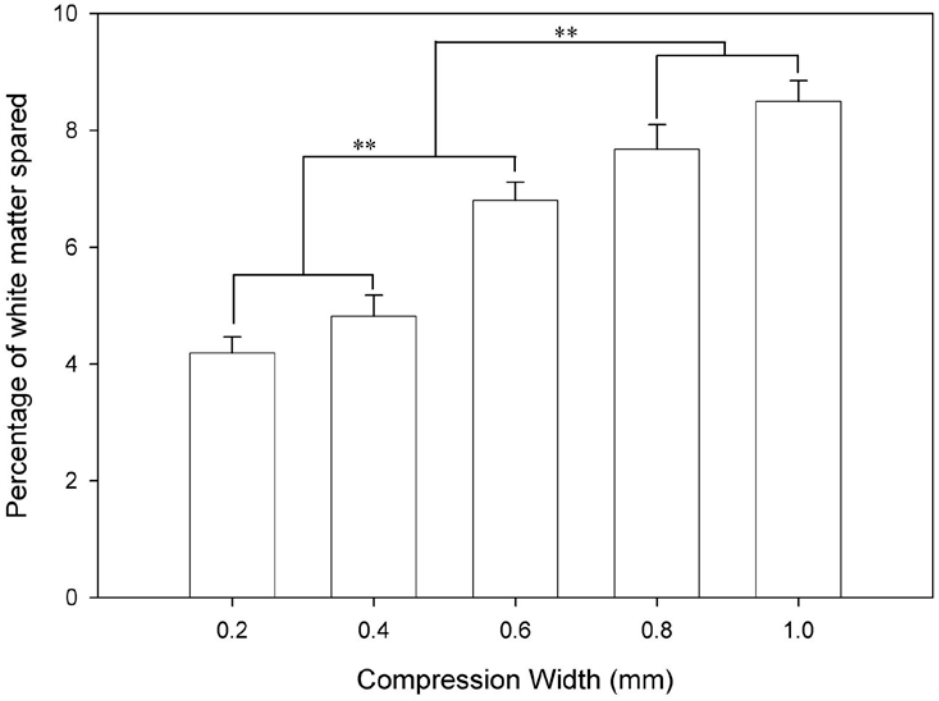


Figure 3-9: White matter sparing significantly decreases with increased injury severity. Data clustered into 3 groups, 0.2-0.4, 0.6 and 0.8-1.0. Each group was significantly different from each other but not within the cluster. White matter quantification strongly correlates with injury severity ($r^2 = 0.980$). Data are expressed as mean ± standard error. ** denotes a significant difference, $P < 0.01$.

3.3.5 Correlations

To help us evaluate the accuracy of spinal injuries induced, correlation coefficients were calculated for each measurement both against the injury severity and each other. The blood GSH measurements were the only assessment where no significant correlations between severities at any time point could be found (Appendix B, Figure B1). The 24 hour sample was the closest to significance in correlation to injury ($r^2 = 0.332$, $P = 0.078$)(Appendix B, Figure B2). All other measurements including: small bladder ($r^2 = -0.566$, $P = 0.002$)(Appendix B, Figure B3), recovery bladder ($r^2 = -0.585$, $P = 0.001$) (Appendix B, Figure B3), BBB scores ($r^2 = 0.663$, $P < 0.001$) (Appendix B, Figure B4), inclined plane scores ($r^2 = 0.652$, $P < 0.001$) (Appendix B, Figure B5) and white matter sparing ($r^2 = 0.950$, $P < 0.001$) (Appendix B, Figure B) showed significant correlation to injury severity.

Additionally, all measurements correlated with white matter sparing: blood GSH ($r^2 = 0.373$, $P = 0.066$), small bladder ($r^2 = -0.508$, $P = 0.010$), recovery bladder ($r^2 = -0.684$, $P < 0.001$), BBB scores ($r^2 = 0.752$, $P < 0.001$) and inclined plane scores ($r^2 = 0.675$, $P < 0.001$). Not surprisingly the two locomotor scores correlated significantly with each other ($r^2 = 0.697$, $P < 0.001$). Recovery of micturition appeared to be the more sensitive of the two bladder measurements as it also correlated with both the BBB ($r^2 = -0.636$, $P < 0.001$) and inclined plane scores ($r^2 = -0.383$, $P = 0.037$). Scatter plots were developed for correlations and can be found in Appendix B.

3.4 Discussion

3.4.1 Short term biochemical changes

Our laboratory has previously demonstrated a decrease in GSH in spinal cord tissue after SCI (Kamencic et al., 2001) and we have demonstrated decreases in both whole blood and spinal cord tissues following aneurysm clip compression. Results in whole blood GSH samples using the forceps compression differed from that using the aneurysm clip. Using the forceps model, there was a significant decrease in blood GSH by 2 hours after injury in the two most severe injuries, animals recovered partially, by 6 hours with a subsequent decreased at 24 hours. Using the aneurysm clip, the decrease in GSH seen at 2 hours was sustained for the full 24 hour period of the study. This discrepancy is likely due to the differences in the models.

The forceps model delivers a medial-lateral compression where as the aneurysm clip model delivers an anterior-posterior compression. This difference in models may affect the severity of vascular disruption during compression as the major arteries supplying the cord are the anterior and posterior spinal arteries. These arteries would be directly compressed in the aneurysm clip model but only indirectly, by distortion of adjacent tissues, and subsequent edema in the forceps model. In addition, the severity between models should be taken into account as BBB scores differ from 2.6 in the aneurysm clip model to 7.3 in the 0.2 mm injury of the forceps model. The decrease in GSH seen did not correlate to the injury severity in a continuous fashion, as the injuries tended to group into two categories: severe (0.2-0.6 mm) and moderate (0.8-1.0 mm). Having the data cluster is a similar finding to that seen in Gruner et al. (1996) who

demonstrated clustering of injury responses into pairs of adjacent injury groups (0.8-1.0, 1.2-1.4 and 1.6-1.8 mm).

The decrease in GSH at the 24 hour time point correlates with the time at which the neutrophil portion of the inflammatory response is peaking (Anderson, 1992) which may account for the decrease seen in GSH at this time point. In addition, there is a significant decrease in mitochondrial respiration rate 24 hours after injury which may contribute to this finding (Sullivan et al., 2007).

3.4.2 Recovery of micturition

No significant changes were seen among individual injury groups in the bladder measurements. When injuries were grouped as severe and moderate significant differences were seen between groups for both small bladder and recovery measurements. A significant correlation was seen between the injury severity and for both types of measurements, similar to that seen in the forceps model in mice (Plemel et al., 2008). Bladder control measurements of micturition also correlated significantly with white matter sparing (r^2 = -0.684 recovery) which is also a similar finding to Plemel et al. (2008) who found an r^2 of -0.626.

The correlation of recovery of micturition with injury severity probably reflects the improved maintenance of white matter across the injury site and therefore the spinal tracts that regulate sacrospinal preganglionic sympathetic neurons responsible for micturition. These tracts, which carry information from the pontine micturition center, are believed to travel in the lateral white matter in both cats (McMahon et al., 1982) and humans (Nathan and Smith, 1958). Partial recovery of micturition spontaneously after injury has been documented previously and has been linked to segmental reflexes (Basso

et al., 1995; Ko et al., 1999). Recovery of coordination, which requires supraspinal input occurs over a longer period of time and is thought to involve the maintenance of bulbospinal projections (Pikov et al., 1998).

Importantly, the focus of SCI research is beginning to shift to better reflect the priorities of spinal trauma patients which include bladder, bowel and sexual function even above that of locomotor ability in the paraplegic population (Anderson, 2004). Therefore, it is important to incorporate measurements, such as this admittedly crude assessment of micturition, into an experiment. These measurements require no additional equipment, are easily integrated into the experimental design and provide potentially important insights into the relationship between injury, therapeutics and micturition.

3.4.3. Behavioral and histological changes

In both behavioral assessments the only significant differences between injury groups were that of the two most severe injuries with the least severe, a difference that was maintained when the data was grouped. This stepwise progression of BBB scores is very similar to that seen by Gruner et al. (1996) and others (Kloos et al., 2005; Plemel et al., 2008). The difference in compression widths from one injury to another may not induce a significant enough difference in overall outcome to differentiate the injuries though behavioral or histological quantifications. Regardless of the stepwise response, the significant correlations between injury severity and both behavioral and histological outcomes indicate the consistency of the stepwise response of the injury model. We believe that one of the strengths of this model is the ability to confirm the compression width both immediately prior to and following use.

In expanding, and partially reproducing the previous rat forceps model (Gruner et al., 1996), it was important to assess the similarities between our findings in order to determine the reproducibility of the model between laboratories. In order to accomplish this, locomotor scores had to be converted from the Gruner et al. (1996) article, which used the Ohio State Motor Scale (Behrmann et al., 1992) (previously correlated to the New York Scale (Saruhashi and Young, 1994)), to that of the BBB scale (Basso et al., 1995). We therefore created a table to cross reference all three scales to easier compare locomotor recovery between scales (Table 3-2).

Secondly, the data within the Gruner paper analyzing white matter sparing was in units of area and therefore was converted by dividing the area of white matter spared in a given injury by the total cross sectional area of the laminectomy control to give us a percentage of spared white matter. As figure 3-10 indicates our data is well in line with that of Gruner et al. (1996) for both locomotor scores and white matter. The differences seen in the BBB scoring may be due to the conversion from the Ohio scale to the BBB scale.

An elegant study recently demonstrated the correlation between the percentage of white matter spared and the subsequent locomotor recovery (Kloos et al., 2005). To demonstrate the similarities between percentage of white matter sparing in our study and BBB scores a scatter plot was created expressing our data, that of Kloos et al. (2005) and Gruner et al. (1996) (Figure 3-11). As this figure shows our white matter and BBB correlates have great similarity to that of Kloos et al, however less so with Gruner et al. It should be noted that the discrepancy with the Gruner data may be a result of the conversion of the Ohio Scale to BBB and the non-linearity of white matter sparing (Kloos et al., 2005).

Table 3-2: Cross reference table used to compare BBB, Ohio State Locomotor scale and New York Locomotor scales.

BBB	NYU	OSU	Description - BBB	NYU/OSU
0	0	0	No observable hind limb movement	
				Active movement of hip and/or knee
1	1-	1	Slight movement in one or two joints	Slight movement at the hip
2	1	2	Extensive movement in 1 joint ± slight movement in another	Active movement at the hip or knee
3	1+	3	Extensive movement of two joints	active movement of hip and knee
4			Slight movement of all 3 joints	
5			Slight movement of 2 joints, extensive movement in 3rd	
				Active hip, knee and ankle movement
6	2-	4	Slight movement of three joints	weak ankle movement
7	2	5	Extensive movement of all three joints	Active movements of all 3 joints
8	2+	6	Sweeping movement with no weight support	Attempts at support are seen
				Active support with stance or uncoordinated gait
9	3-	7	Plantar placement of paw, weight support in stance only	Support in stance only
10			Occasional weight-supported plantar steps	
11	3	8	Frequent to consistent weight supported plantar steps	Active support, uncoordinated gait
12	3+	9	Frequent to consistent weight supported plantar steps, occasional FL-HL coordination	Occasional bouts of coordinated gait
			Frequent to consistent weight supported plantar steps +	*Coordinated gait +*
13	4-	10	Frequent FL-HL coordination	Lack of control of foot and ankle
14	4	11	Consistent FL-HL coordination, rotated paw placement	Coordinated fore- and hindlimb gait, wide support base
15	4+	12	Consistent FL-HL coordination, rotated paw placement, no toe clearance	Narrowing support base, no abdomen drag, some toe drags
			Consistent FL-HL coordination +	*Normal or almost normal gait +*
16	5-	13	Parallel paw placement at contact – rotated at lift off, toe clearance frequently	Occasional toe drags, slight unsteadiness on full speed turns
17			Parallel paw placement at contact and lift off, toe clearance frequently	
18			Parallel paw placement at contact and rotated at lift off, toe clearance consistantly	
19			Parallel paw placement at contact and at lift off, toe clearance consistently, tail down sometimes	
20			Parallel paw placement at contact and at lift off, toe clearance consistently, tail consistently up, trunk instability	
21	5	14	Parallel paw placement at contact and at lift off, toe clearance consistently, tail consistently up, trunk stable	Steady gait, no toe drags, full speed turns

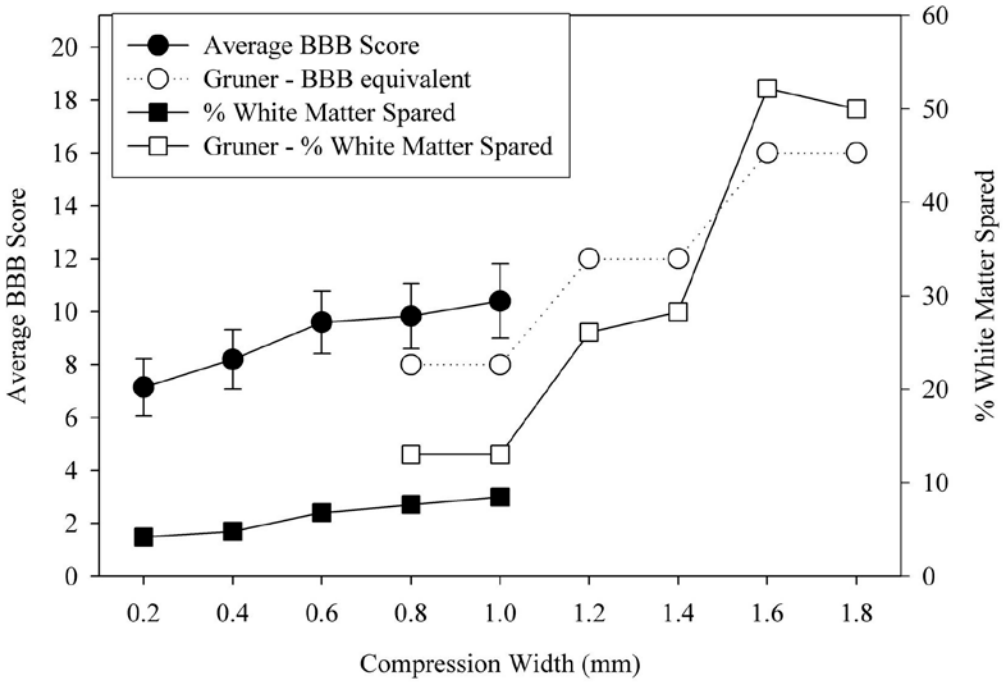


Figure 3-10: Correlation of white matter and locomotory scores with Gruner et al. (1996). BBB scores from the current study correlated well with converted Ohio State Motor Scale scores. Percentage white matter sparing also correlated well with Gruner. Our data are expressed as average ± standard error, data from Gruner et al. is expressed as mean only.

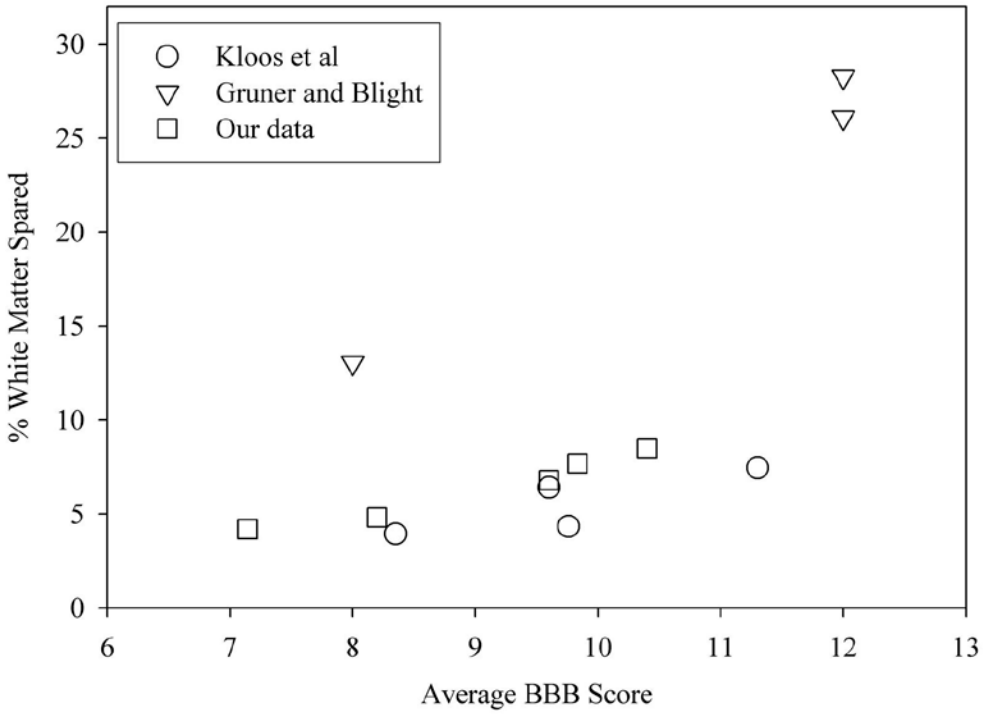


Figure 3-11: Scatter plot demonstrating the correlation between white matter and BBB scores for the forceps model and the NYU impactor. All data are expressed as mean only.

Correlations coefficients for injury severity and BBB scores (r2 = 0.663) compare well with that of Gruner (r2 = 0.641), however a stronger correlation was found between white matter sparing and injury in our experiments (r2 = 0.980 vs r2 = 0.473). In addition, open field testing scores and white matter sparing correlated strongly in our study (r^2 = 0.752, P < 0.001), Gruner et al. (1996) (r^2 = 0.704) and most recently in the mouse forceps model (r^2 = 0.781) (Plemel et al., 2008).

3.4.4 Conclusions

It is essential that SCI models induce a consistent injury (Behrmann et al., 1992; Behrmann et al., 1994) which mimics the clinical situation (Behrmann et al., 1992; Tator, 2006). Models should also allow for rapid turn over of animals and the ability to modify the injury severity in order to assess mild to severe injuries with a single apparatus. By extending the initial study describing the forceps compression for mild to moderate injuries (Gruner et al., 1996), we have demonstrated the validity of the forceps compression model in the moderate to severe injury range in rat. This model contains characteristics of the clinical traumas seen including: disruption of vascular supply (Khan and Griebel, 1983a) and compression of the spinal column as seen following vertebral dislocation (Tator, 1983). In addition, the forceps model has the advantages of being low cost, able to produce a graded injury and most importantly, injury severity can be qualified both immediately prior to and after use to ensure accurate and consistent injury.

CHAPTER 4.0

GLUTAMINE INDUCES POTENT NEUROPROTECTION FOLLOWING SPINAL CORD INJURY IN RATS

4.1 Introduction

The pathophysiology of SCI evolves as a combination of phases of damage termed the primary and secondary phases. The primary phase involves the initial mechanical damage to the spinal cord tissue by compression, contusion, laceration and/or shearing. Although this initiates the injury, it is thought to comprise only 10% of the overall injury to the tissue (Young et al., 1982). The secondary phase that immediately follows can last for days, weeks or months and is a highly complex process involving numerous inter-related mechanisms including: ischemia, oxidative stress, inflammation, excitotoxicity and apoptosis which have all been extensively reviewed (Kakulas, 1987; Anderson, 1992; Juurlink and Paterson, 1998; Tator, 1998; Kwon et al., 2004; Norenberg et al., 2004; Profyris et al., 2004; Hagg and Oudega, 2006; Trivedi et al., 2006).

Of the many mechanisms affecting the amount of secondary damage, one of the key factors is oxidative stress (Aksenova et al., 2002; Bao and Liu, 2002; Xu et al., 2005) and resultant inflammation (Jones et al., 2005; Fleming et al., 2006). Two of the more well known antioxidants, vitamins C and E have been studied for therapeutic potential with findings of improved blood flow and decreased lipid peroxidation (Hall et al., 1989; Iwasa et al., 1989; Taoka et al., 1990; Katoh et al., 1996; Wang et al., 2006). In addition, there have been many other compounds that work through antioxidant pathways that have shown to be beneficial (Kaptanoglu et al., 2002; Hillard et al., 2004;

Kayali et al., 2005; Sharma et al., 2006) including previous research from our laboratory (Kamencic et al., 2001; Schultke et al., 2003). Although many compounds have been studied in the laboratory setting, few have made a successful transition to clinical trials with maintained efficacy (Gilgun-Sherki et al., 2002).

Therapeutics that have a broad spectrum of effects in various tissues and organ systems ought to improve outcome after SCI. The focus of our laboratory has been to supplement the stressed organism with compounds that will help the body's endogenous anti-oxidant defense systems cope with the oxidative stress due to injury. The major approach has been to enhance maintenance of GSH levels in the injured tissues.

GSH is the most potent endogenous antioxidant in the body and is essential for the scavenging of peroxides and recycling oxidized vitamin E (Rose and Bode, 1995). Peroxide scavenging is essential to minimizing oxidative stress in living tissues as peroxides are produced under normal physiological states. The enzyme catalase can contribute to the scavenging of hydrogen peroxides; however, it does so with low affinity and is unable to scavenge organic peroxides unlike GSH peroxidase (GPx) (Simmons and Jamall, 1988). The principle mechanism of peroxide scavenging is through the enzyme GPx which uses GSH as the electron donor (Flohe, 1978). Lipid peroxides and their breakdown products which have been repeatedly demonstrated to be detrimental in nervous tissue (Keller et al., 1997; Mark et al., 1997; Springer et al., 1997a; Baldwin et al., 1998; Picklo et al., 1999) can be scavenged by GSH-dependent glutathione peroxidase or by the formation of GSH adducts through the enzyme GSH S-tranferase (Gulick and Fahl, 1995). GSH is a tripeptide that is synthesized in two steps (Figure 4-1) (Meister, 1988). In step one the enzyme GCL forms γ-glutamyl-cysteine from glutamate and cysteine. The second step is catalyzed by GSH synthase where

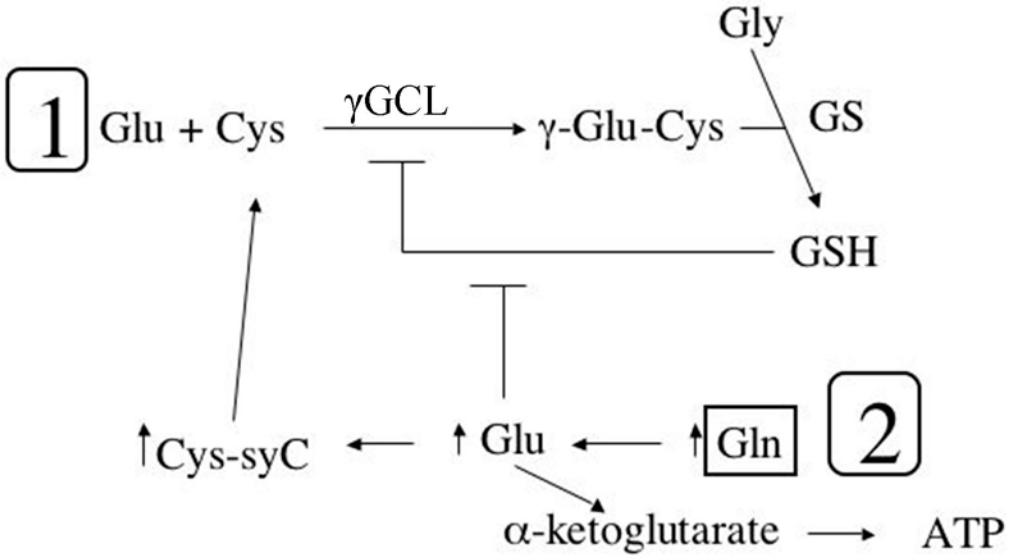


Figure 4-1: Cartoon outlining the regulation of glutathione (GSH) synthesis. Equation 1 running from left to right demonstrating that the first step in GSH synthesis is the ligation of glutamate (Glu) to cysteine (Cys) to form the dipeptide g-glutamyl-cysteine (γ-Glu-Cys) catalyzed by the enzyme γ-glutamyl-cysteine ligase (γGCL). Cysteine is the rate-limiting amino acid in this first step of synthesis. The second step of synthesis is the ligation of glycine (Gly) to γ-Glu-Cys to form GSH. GSH has feedback inhibition on γGCL activity that limits cellular GSH concentrations. Equation 2 running from right to left outlines the therapeutic possibility of glutamine (Gln). Glutamine is converted to glutamate by the enzyme glutaminase. Glutamate influences GSH synthesis in two manners. Firstly, in alleviates the inhibition of γGCL activity by GSH and secondly, it promotes the uptake of cystine (Cys-syC) by the glutamate-cystine antiporter. Cystine is reduced to cysteine, the rate-limiting amino acid in GSH synthesis. In addition, glutamate can be converted to α-ketoglutarate promoting the production of ATP in compromised cells.

glycine is ligated to γ-glutamyl-cysteine. Step one which provides the substrate for step two is rate-limiting in several ways including the availability of cysteine and GSH-mediated negative feedback loop affecting GCL (Meister, 1988). Although cysteine is the rate-limiting amino acid, intracellular glutamate concentrations can affect the synthesis of γ-glutamyl-cysteine in two ways.

Most of the extracellular cysteine is oxidized to cystine: in the CNS cystine is taken up into cells (mainly astrocytes) by the cystine/glutamate antiporter (McBean and Flynn, 2001). As intracellular concentrations of glutamate increase so will the activity of the antiporter and consequently intracellular concentrations of cystine which can then be reduced to cysteine thereby promoting GSH synthesis. GSH, itself, has a feedback inhibition of GCL that is alleviated by glutamate (Meister, 1988); hence, an increase in intracellular glutamate should also increase GSH synthesis by this second mechanism. In principle, one should be able to increase intracellular glutamate by administering the amino acid glutamine since intracellular glutamine is acted upon by glutaminase to give rise to glutamate and ammonia (Kvamme et al., 2001).

Of relevance to SCI is that glutamine increases the content of GSH in a number of contexts (Hong et al., 1992; Harward et al., 1994; Denno et al., 1996; Flaring et al., 2003). Our laboratory has previously demonstrated the ability to improve the maintenance of GSH at the site of SCI using the procysteine compound L-2-oxothizolidine-4-carboxylate (OTZ). By improving GSH concentrations, both locomotor function and white matter sparing was improved by 6 weeks post injury (Kamencic et al., 2001). Furthermore, the concentration of GSH has been associated with improved maintenance of blood brain barrier (Agarwal and Shukla, 1999) and decreases in loss of

tissue, motor function and decreased signs of lipid peroxidation and pro-apoptotic factors following SCI (Genovese et al., 2007).

In addition to increasing GSH, glutamine is being widely examined now for its therapeutic potential due to the multifaceted nature of its effects. Glutamine functions metabolically as a nitrogen donor for protein synthesis (Zalkin and Smith, 1998), a carbon donor for the tricarboxylic acid (TCA) cycle (Tildon and Roeder, 1984), an important substrate for ammoniagenesis (Halperin and Bun-Chen, 1987), a substrate for neurotransmitters such as GABA (Peng et al., 1993) and an essential energy source for proliferating lymphocytes (Ogle et al., 1994; Chang et al., 2002; Peng et al., 2006a).

Although traditionally considered a nonessential amino, there is a current consensus to reclassify glutamine as 'conditionally essential' in states of physiological stress (Lacey and Wilmore, 1990). Glutamine concentrations have been demonstrated repeatedly to be decreased following many types of trauma (Engel et al., 2003), burns (Parry-Billings et al., 1990; Gore and Jahoor, 1994), surgery (Blomqvist et al., 1995; Hammarqvist et al., 1996), and poor nutrition states (Van Der Hulst et al., 1994). Following SCI, plasma glutamine concentrations have been shown to decline in humans (Rogeri and Rosa, 2005) and in both plasma and muscle in SCI rats (Tanhoffer et al., 2007). Decreases in plasma glutamine have been linked to immunodepression (Parry-Billings et al., 1990), increased muscle catabolism (Kuhn et al., 1999) and decreased GSH content (Flaring et al., 2003).

Supplementation with glutamine has proven beneficial for a wide variety of conditions including following multiple trauma (Houdijk et al., 1998; Houdijk and van Leeuwen, 2000; Bakalar et al., 2006; Dechelotte et al., 2006), bone marrow transplant (Brown et al., 1998; Schloerb and Skikne, 1999; Coghlin Dickson et al., 2000; da Gama

Torres et al., 2008), cardiac muscle ischemia (Khogali et al., 1998) and radio- and chemo-therapy (Rouse et al., 1995; Yoshida et al., 2001; Savarese et al., 2003). Studies examining critically ill patients however, have shown no positive influence on length of hospital stay (Kumar et al., 2007), infectious morbidity (Schulman et al., 2006) or mortality (Hall et al., 2003; Schulman et al., 2005; Kumar et al., 2007).

4.2 Materials and methods

4.2.1 Modifying forceps for use in spinal compression injury surgery

See Section 3.2.1

4.2.2 Animals

See Section 2.2.2

4.2.3 Surgery and tissue collection

See Section 2.2.2 and 2.2.3

4.2.4 Blood GSH measurements

Blood collections occurred during the surgical preparation (-1 hour) and 1, 6, 24, 48 and 72 hours after the time of glutamine/vehicle administration (1 hour post-surgery). Blood was collected as per protocols found in Section 2.2.4.2

4.2.5 Evaluation of bladder function

See Section 3.2.5

4.2.6 Testing of functional performance

See Section 2.2.5

4.2.7 Histology

See Section 2.2.6

4.2.8 Experimental design

Our project was comprised of two main experiments. The first objective was to assess the potency of glutamine treatment at a range of injury severities. For our first experiment, 88 male Wistar rats were randomly divided into 11 experimental groups (N = 8), two treatment groups (saline and glutamine) for each level of severity being examined (0.2, 0.4, 0.6, 0.8 and 1.0 mm) and a sham saline treated group. All animals received injections of saline or glutamine 1 hour post-surgery and every 12 hours for 1 week.

The second objective was to determine the optimal concentration for glutamine at the injury severity showing the highest potency of glutamine. This experiment involved 42 male Wistar rats that were randomly divided into 7 experimental groups (N = 6), one sham, one saline treated group, one 1 mmol/kg alanine group and one each for a range of glutamine concentrations (0.5, 1, 2.5 and 5 mmol/kg).

4.2.9 Statistical analysis

With the exception of blood GSH, all data from individual injury groups were analyzed using a 1-way ANOVA and a post-hoc Tukey's test. Blood GSH measurements were assessed for significance using a 3-way ANOVA with post-hoc Tukey's. Differences were considered significant if the P value < 0.05. Pearson's correlation coefficients were calculated for each data set and correlations were considered significant if $P < 0.05$. With the exception of the micturition data, which

were expressed as mean ± standard deviation, all other data was expressed as mean ± standard error.

4.3 Results

4.3.1 Experiment #1 examining various injury severities

4.3.1.1 Blood GSH concentrations following SCI

In saline treated animals, by 2 hours post-injury, there was a significant decrease in blood GSH in the 0.4 mm injury animals (0.582 ± 0.014 vs 0.606 ± 0.014, P = 0.036) which was not seen in response to the milder injuries (Figure 4-2). Collectively, the lowest concentrations of GSH were seen in the 24 hour samples when the most severe injuries (0.2 - 0.6 mm) had significantly lower concentrations of GSH than the pre-surgical blood samples (Figure 4-2 A-C). Milder injuries (0.8 - 1.0 mm) saw no significant decreases in GSH at any time point and there were no significant decreases in GSH after the 24 hour time point in any injury group. In animals that underwent the sham surgery there were no significant decreases in blood GSH at any time point (see Table 3-1).

Glutamine treatment not only prevented the decrease in GSH but significantly increased it above that of the pre-surgical values at the one hour time point after 0.4 mm injury (Figure 4-2 B) (0.638 ± 0.011 vs 0.606 ± 0.014, P = 0.014). Glutamine treatment also prevented the significant decreases in GSH in the 0.4 mm and 0.6 mm injuries at the 24 hour time points (Figure 4-2 B,C). Even in the milder injuries (0.8 - 1.0 mm) where no significant decreases were seen, glutamine treatment increased GSH concentrations above that of the saline treated 48 hours after injury (Figure 4-2 D,E).

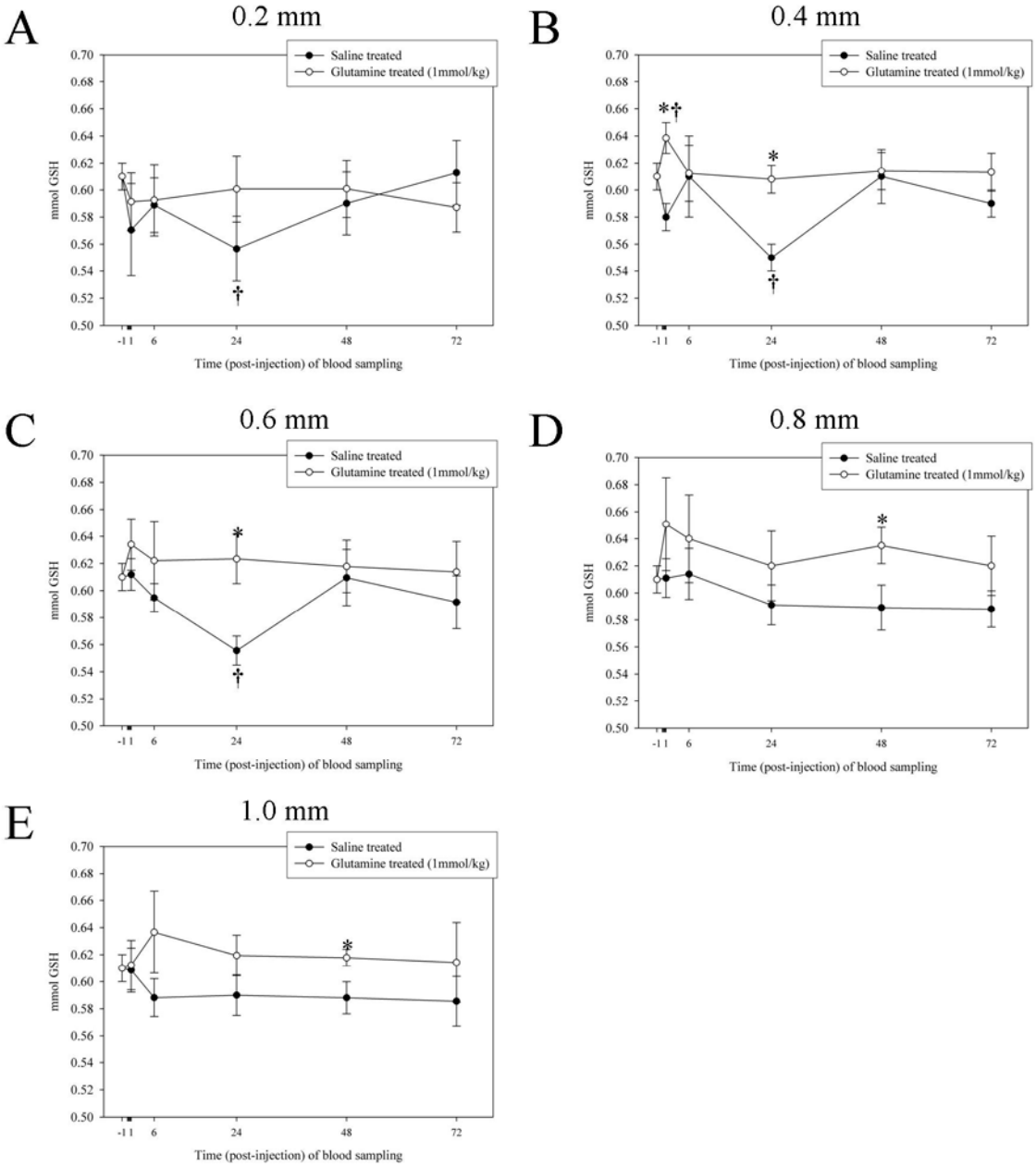


Figure 4-2: Glutamine administration improved maintenance of whole blood glutathione (GSH) concentrations at various time points following spinal cord injury. † denotes a significant difference ($P < 0.05$) from pre-surgical values. * denotes a significant difference from coordinate saline treated sample. Data are expressed as mean ± standard error.

4.3.1.2 Evaluation of bladder function

All animals recovered micturition within the 6 week time frame of this study and sham animals did not require any manual bladder expression. As discussed in Chapter 3 the rate of recovery of micturition significantly correlated with the severity of injury for both small bladder (r^2 = -0.566, P = 0.002) and recovery measurements (r^2 = -0.585, P = 0.001). No significant differences were seen between saline treated injury groups (Figure 4-3). Glutamine treatment created only one significant change which occurred in the 0.4 mm injury group, glutamine treatment significantly decreased the number of days until the recovery of micturition compared to saline control (7.8 ± 2.2 vs 10.5 ± 1.8, P = 0.042).

When injuries were grouped as either moderate (0.8 and 1.0 mm) or severe (0.2 - 0.6 mm) no significant differences were found between saline and glutamine treated groups (Figure 4-4 A). When the data were collapsed across injuries significant differences were seen in both partial and complete recovery of micturition (Figure 4-4 B). Glutamine treated animals recovered faster compared to saline treated both using partial (4.6 ± 1.1 vs 6.5 ± 1.2, P = 0.035) and complete (7.1 ± 1.6 vs 10.4 ± 1.3, P = 0.011) recovery of micturition.

4.3.1.3 Locomotory recovery

All sham animals achieved and maintained a BBB score of 21 and an inclined plane score of 55 degrees over the course of the 6 week experiment. As described previously, BBB scores correlated strongly with injury severity and white matter sparing in saline treated animals. Glutamine treatment significantly improved the BBB scores 6 weeks after surgery in animals injured with 0.4, 0.6 and 0.8 mm compression widths

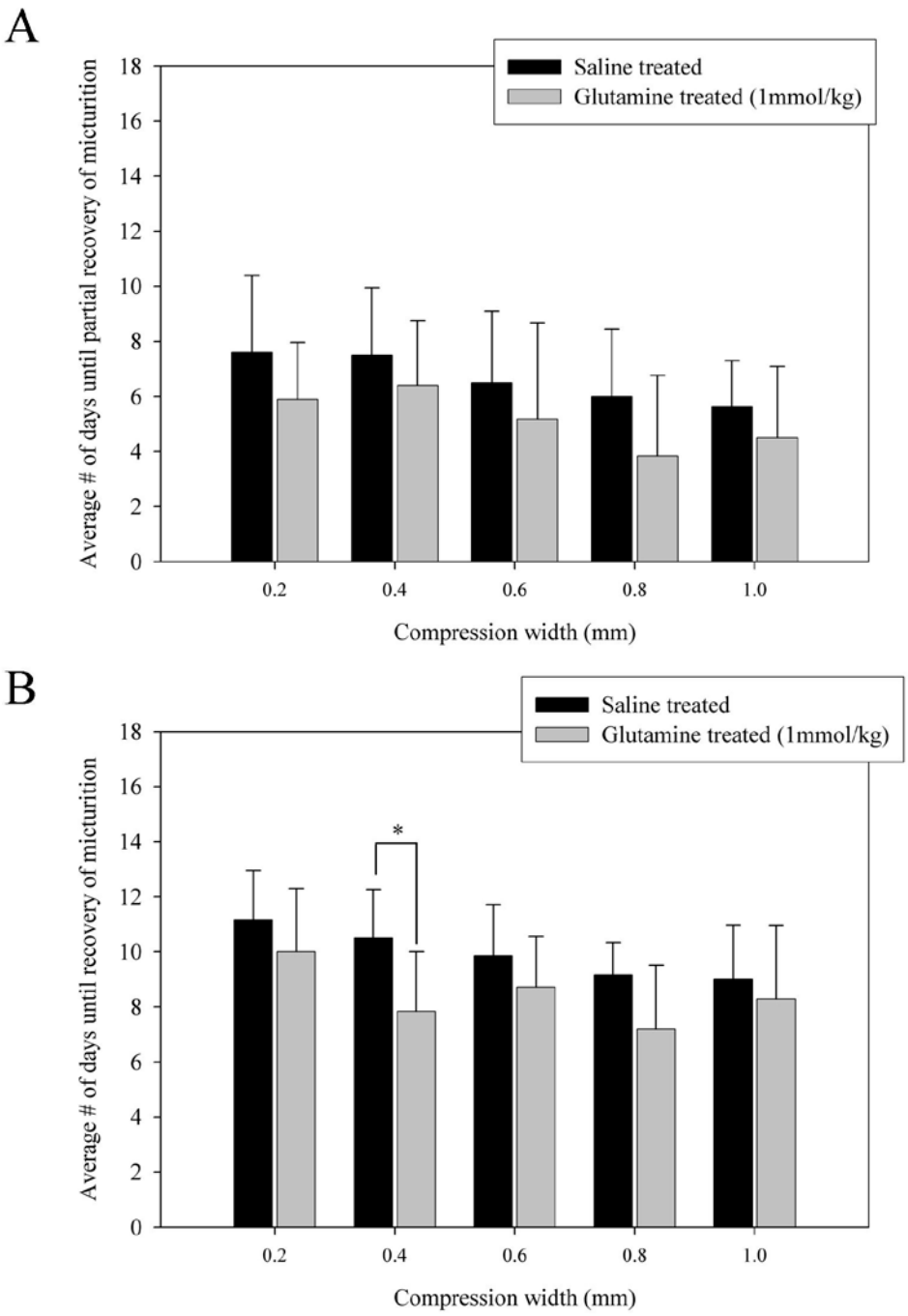


Figure 4-3: The rate of recovery of micturition was increased with glutamine treatment. A) Partial recovery of micturition as indicated by a small bladder size, B) Recovery of micturition. * denotes a significant difference between groups ($P < 0.05$). Data are expressed as mean ± standard deviation.

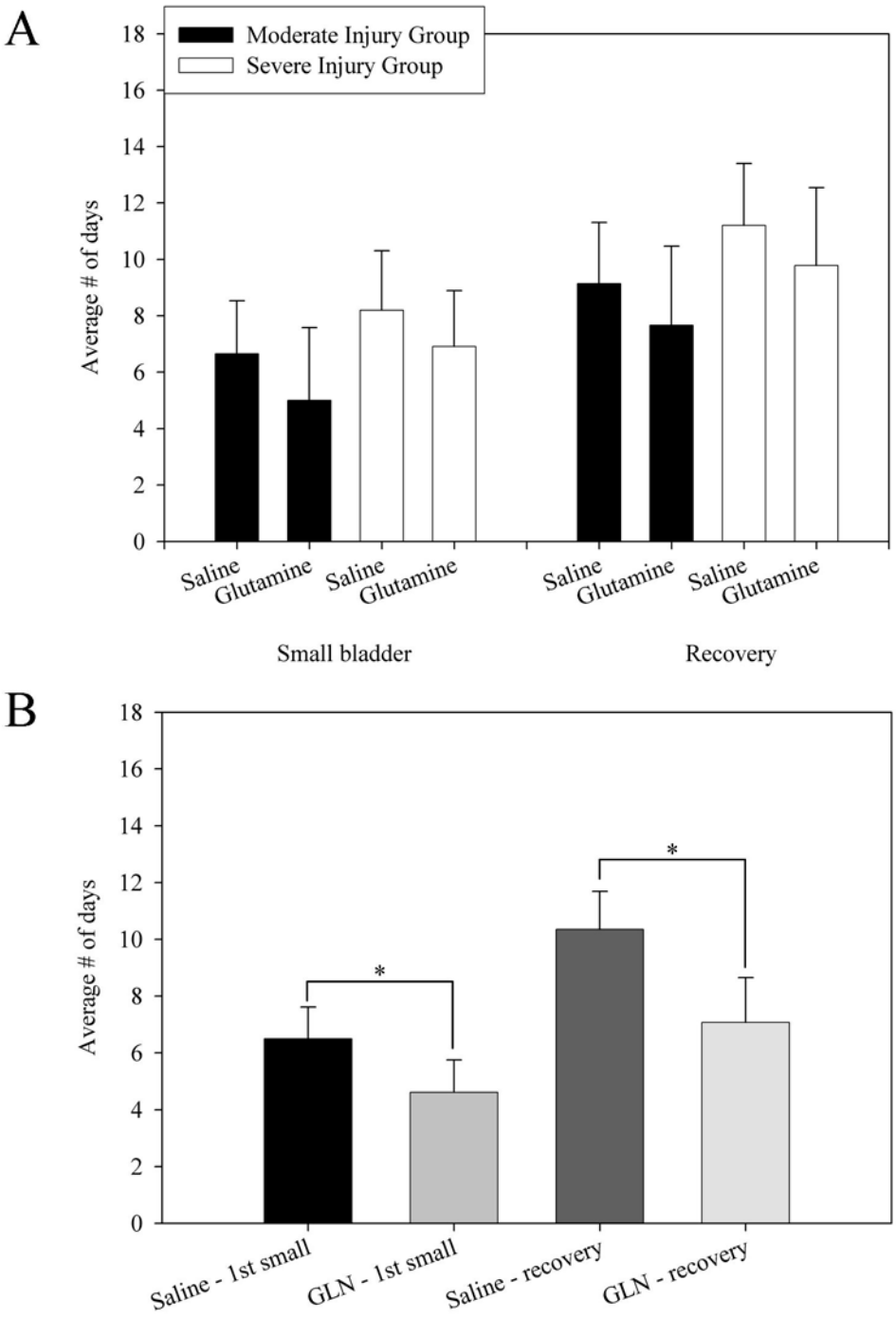


Figure 4-4: The rate of recovery of micturition analyzed by collapsing across injury groups. A) Injuries are grouped into moderate (0.8 - 1.0 mm) and severe (0.2 - 0.6 mm) showing data for both partial (small) and complete recovery of micturition, B) Data are collapsed across injuries showing both data for both partial (small) and complete recovery of micturition. * denotes a significant difference between groups ($P < 0.05$). Data are expressed as mean ± standard deviation. GLN - glutamine

(Figure 4-5). The potency of the glutamine treatment clearly plateaued as the significant improvement seen in the 0.4 mm injured animals was not extended to the milder 1.0 mm injury. The most potent effect of glutamine treatment was seen in the 0.4 mm injured animals. In this group, BBB scores significantly increased ($P < 0.001$) from 8.57 ± 1.12 in the saline treated animals to 13.67 ± 0.42 in the glutamine treated animals.

When examining landmarks of locomotor function, glutamine treatment increased the number of animals who regained the "extensive movement of all three joints of the hind limb" (BBB = 7) and "plantar placement of the paw with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping" which coincides with a BBB score of 9 (Basso et al., 1995). Consistently more animals achieved a BBB score of 7 or better in the glutamine treated group compared to the saline treated, particularly in the 0.4 mm injuries which had only 4 saline treated, but 8 glutamine treated animals reach or exceed this score. In the 0.2 and 0.4 mm injury groups, glutamine treatment doubled the number of animals achieving a score of 9 or higher from 2 to 4 and 3 to 6 respectively.

Similar to the BBB scores, inclined plane scores also significantly correlated with injury severity. However, unlike the BBB scores, glutamine only significantly increased the inclined plane scores in one injury group, the 0.4 mm from 31.4 ± 1.4 in the saline treated to 44.2 ± 1.3 ($P = 0.023$) (Figure 4-6).

4.3.1.4 Histological analysis

No detectable damage to the spinal cord was seen in control animals which underwent laminectomy alone. As discussed previously, a strong significant correlation was found between white matter sparing at the site of injury and the injury severity. All

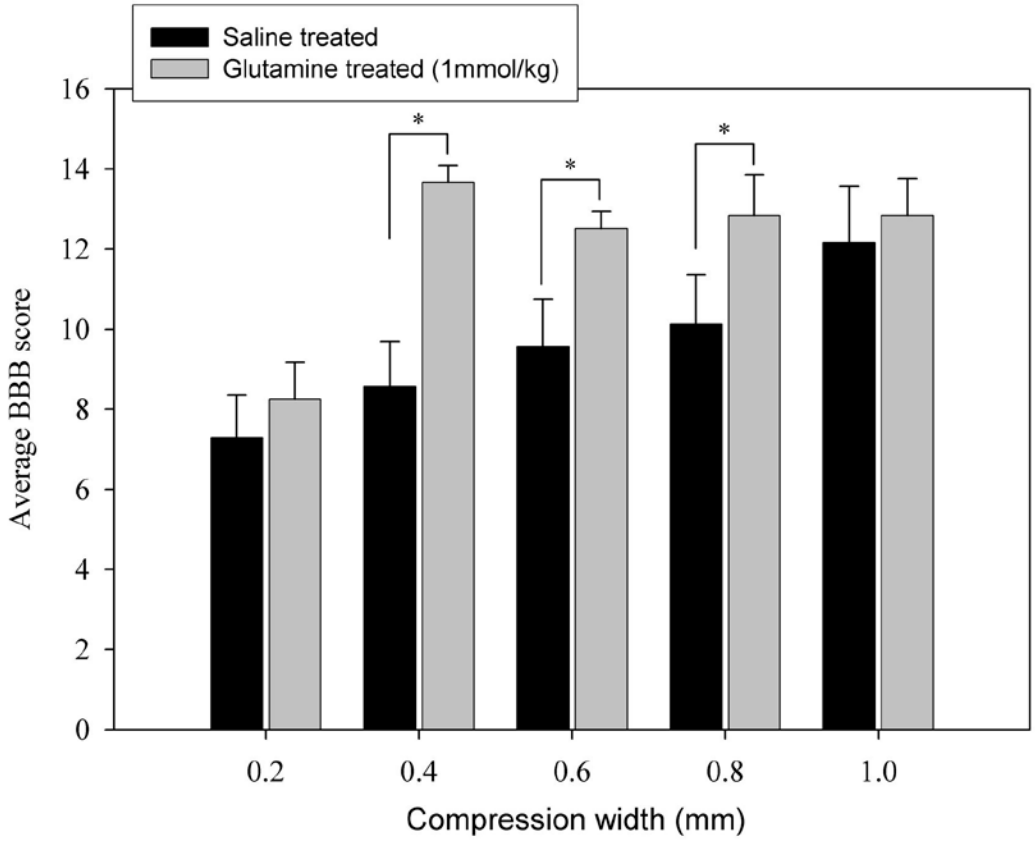


Figure 4-5: Average BBB scores 6 weeks after injury are increased significantly with glutamine treatment across three injury severities. Data shown are the measurements taken 6 weeks after injury and are expressed as mean ± standard error. * denotes significant difference ($P < 0.05$).

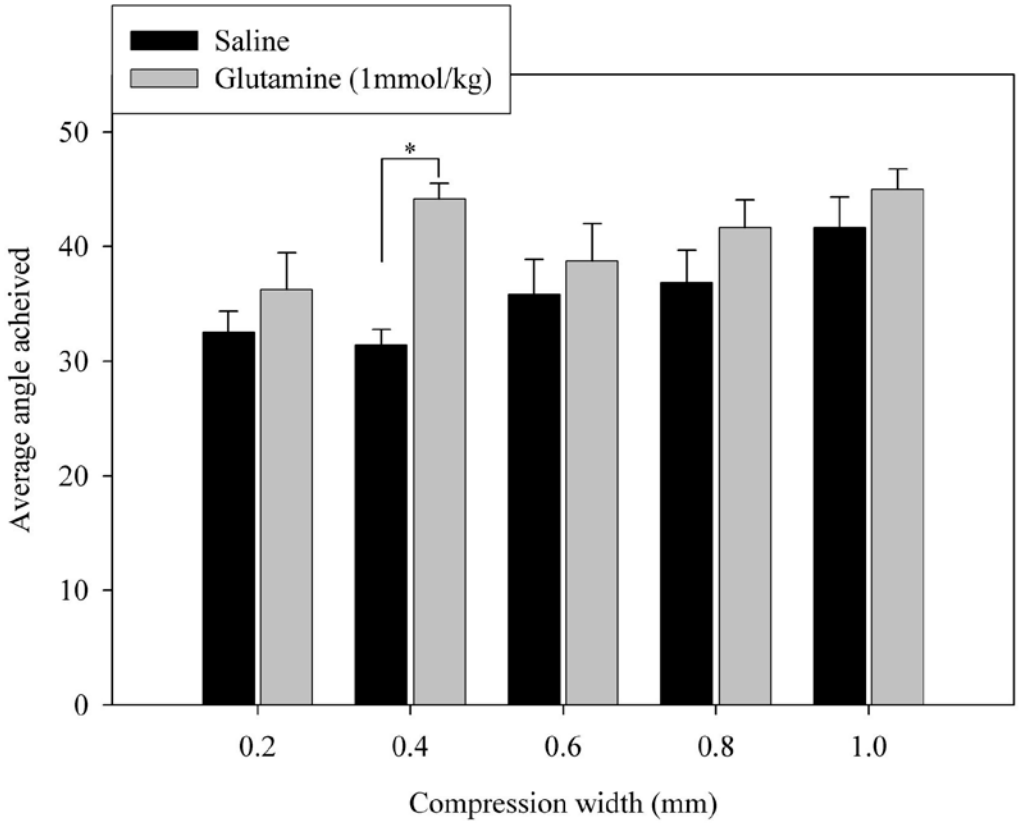


Figure 4-6: Average inclined plane scores are increased significantly with glutamine treatment. Data shown are the measurements taken 6 weeks after injury and are expressed as mean ± standard error. * denotes significant difference ($P < 0.05$).

injured tissues demonstrated a significant loss of both white and gray matter (Figure 4-7 A-J). Classical signs of SCI include microcysts, cell infiltrates and large cystic cavities were commonly seen in injured tissues (Behrmann et al., 1992; Basso et al., 1996; Gruner et al., 1996; Kloos et al., 2005). Consistently, glutamine treated animals had better overall maintenance of tissue morphology and an increased amount of tissue spared regardless of injury severity. Tissue morphology also differed between the saline and glutamine treated animals. Glutamine treated tissues had large cystic cavities which appeared to contain less tissue or cellular debris than the saline treated, furthermore, the degree of cellularity and the amount of scar tissue was also decreased in the glutamine treated compared to the saline treated (Figures 4-8 A-D).

Gray matter within the dorsal horn was seen only in the 0.6 - 1.0mm injuries in saline treated animals but it could be seen in even the 0.2 and 0.4 mm injured spinal cords in the glutamine treated animals. Spared white matter typically was arranged as a superficial rim with a variable depth depending on the amount spared. As discussed previously, the two main locomotor tracts showed some sparing in the saline treated animals with the milder injuries. Areas of white matter containing the corticospinal tract (CST) and rubrospinal tract (RST) within the spinal cord were more often spared in the glutamine treated animals compared to the saline control. The largest difference was seen in the 0.4 mm injury group where 2 and 0 of the saline treated and 7 and 5 of the 8 glutamine treated animals saw RST and CST sparing respectively.

White matter sparing in saline treated animals correlated strongly and significantly with injury severity. Glutamine treatment significantly increased the amount of white matter spared at the epicenter regardless of injury severity (Figure 4-9). Similar to the BBB and inclined plane scores, the potency of glutamine treatment

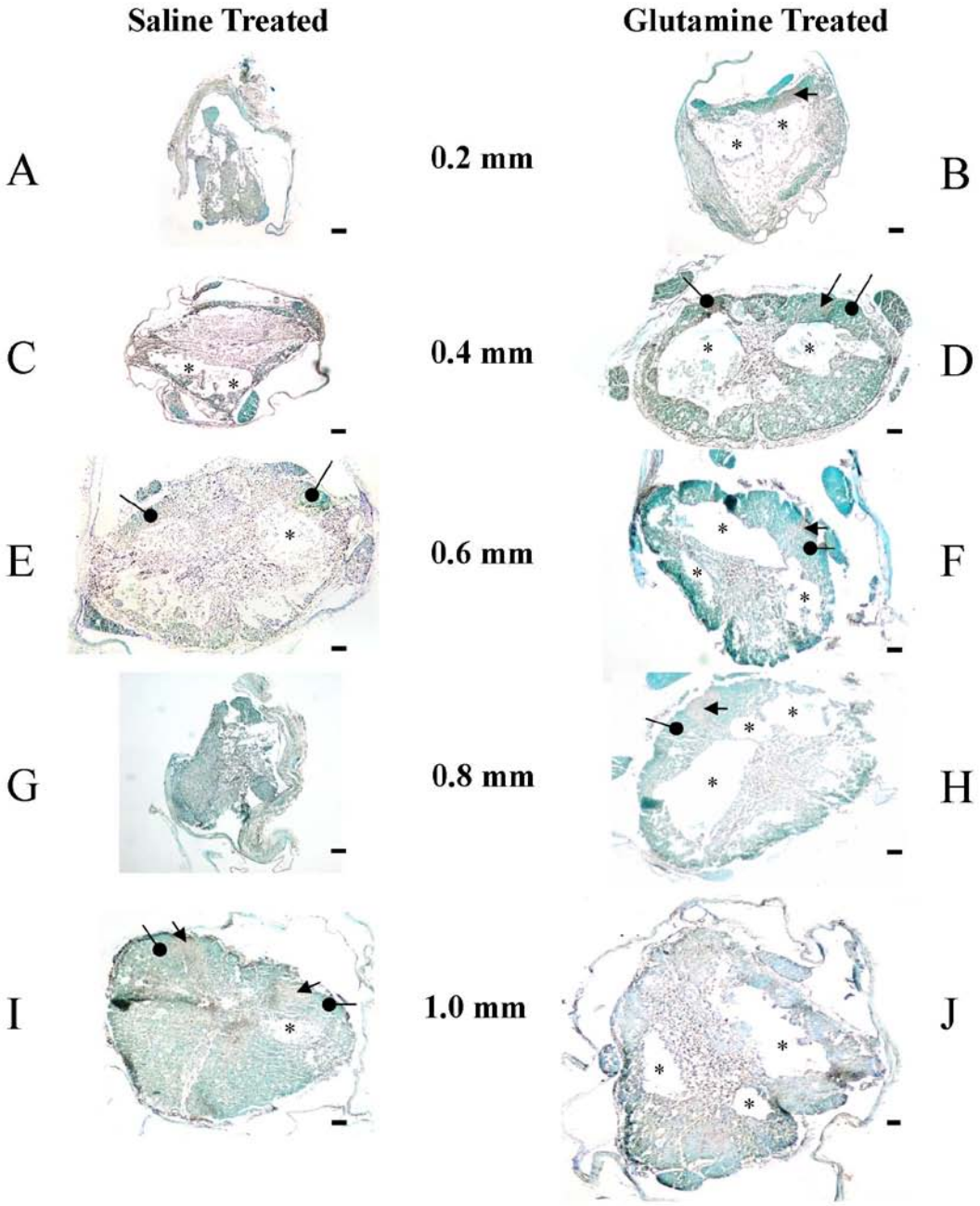


Figure 4-7: Representative histology from animals receiving spinal compression widths ranging from 0.2 - 1.0 mm and treated with saline (A, C, E, G, I) or 1.0 mmol/kg glutamine (B, D, F, H, J). Intact areas of the rubrospinal tract are indicated with round-ended arrows, black pointed arrows indicate areas of spared tissues within the dorsal horns and * denotes areas of cystic cavities. Scale bar represents 100 µm.

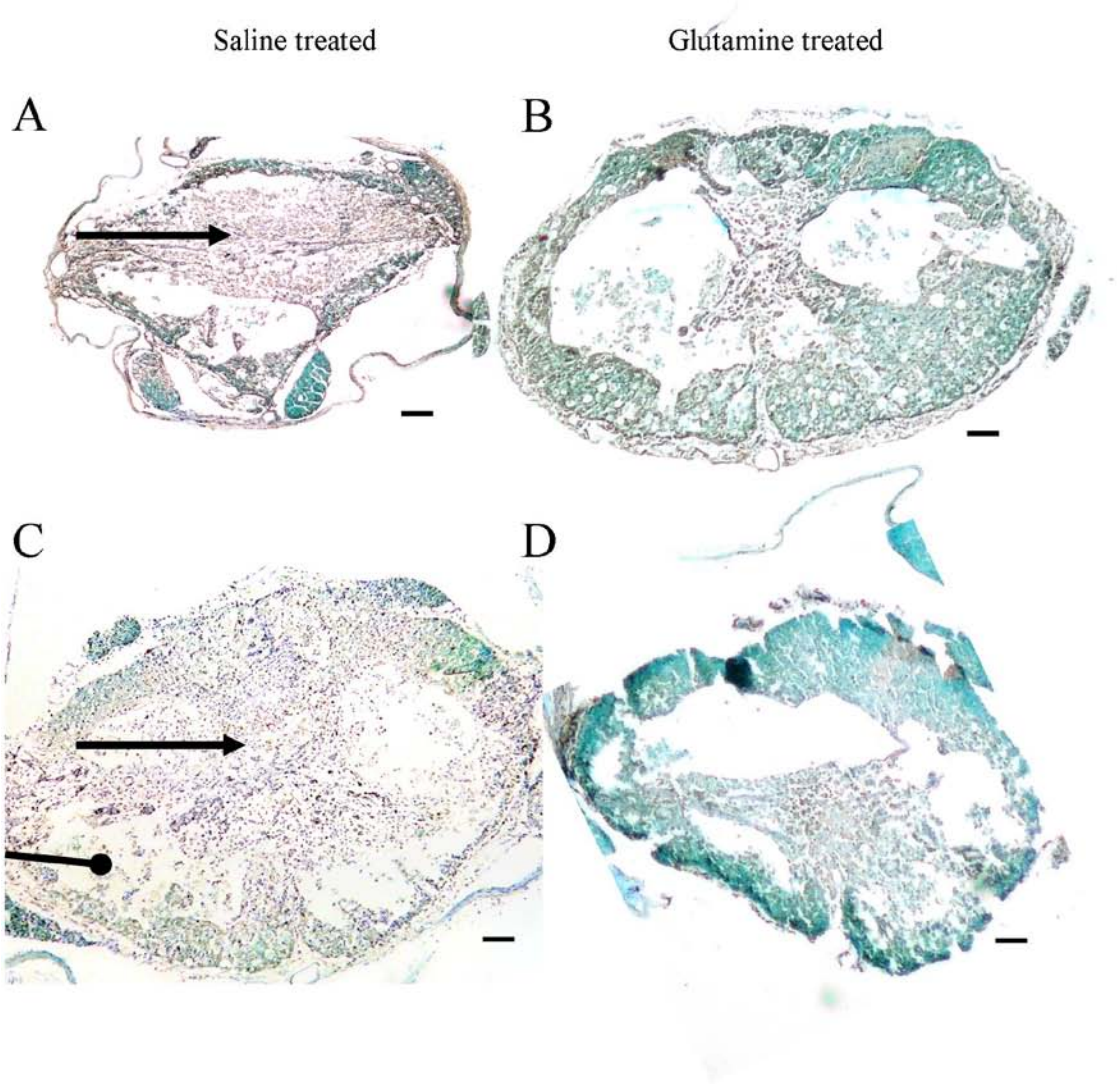


Figure 4-8: Glutamine decreased appearance of scar tissue and debris in epicenter of injury in spinal cord sections taken 6 weeks after injury. A & B are animals that underwent the 0.4 mm compression while C & D are animals that underwent the 0.6 mm compression. Round ended arrows indicate cavities with debris. Arrows indicate areas of scar tissue Scale bar represents 100 μm.

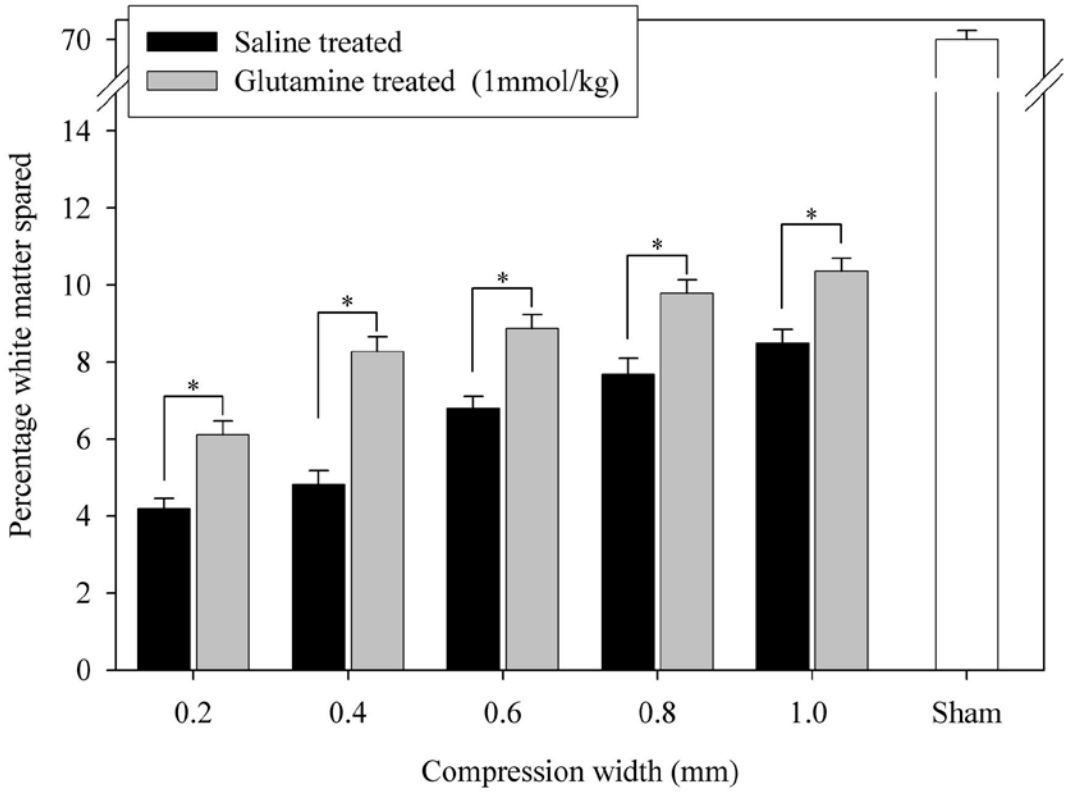


Figure 4-9: Glutamine treatment significantly increased white matter sparing regardless of injury severity. Sham measurements were significantly higher than all injured groups. Data are expressed as mean ± standard error. * denotes a significant difference, $P < 0.01$.

appeared to peak at in the 0.4 mm injured animals with a 72 % increase over saline. This substantial change was not seen in the 0.2 mm injury group (46 %) nor was it seen in the milder (0.6 - 1.0 mm) injuries which saw a ~ 26 % increase in white matter sparing with glutamine treatment. The highest amount of white matter spared in injured animals was in the glutamine treated 1.0 mm injury group (10.36 ± 0.33 %). The lowest of the glutamine treated animals was seen in the 0.2 mm injury group (6.11 ± 0.36 %) which is a similar white matter amount to between the 0.4 and 0.6 mm injuries in the saline treated animals.

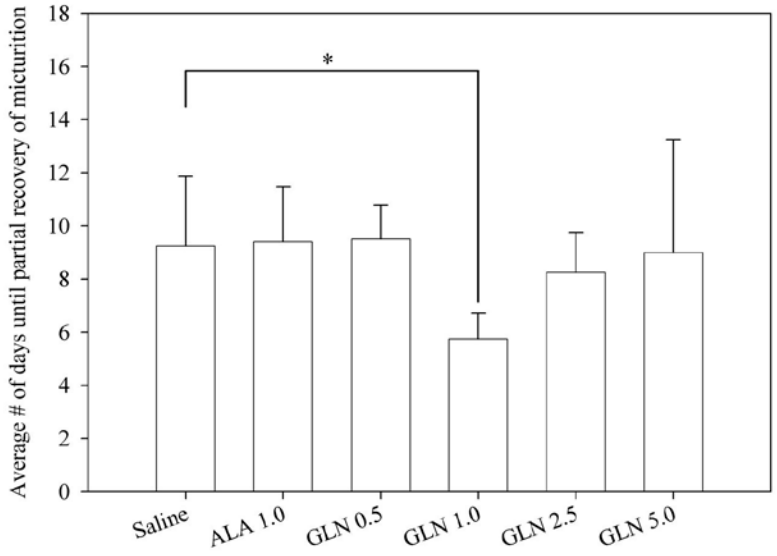
4.3.2 Experiment #2: Examining the effect of various glutamine doses on recovery following SCI.

For this experiment the 0.4 mm compression width was chosen as it demonstrated characteristics of a severe SCI but yet glutamine treatment was able to improve recovery. The concentrations of glutamine used ranged from 0.5 - 5 mmol/kg and alanine (1 mmol/kg) was used as an amino acid control to determine if the effect of the glutamine treatment was due to a non-specific response to amino acid supplementation. Alanine was the amino acid of choice because, similar to glutamine, it plays a role in gluconeogenesis. Alanine contributes to glucose production through the liver compared to renal glutamine. Alanine treatment showed no significant effects in any measurement taken.

4.3.2.1 Evaluation of bladder function

Using both measurements (small and recovery) a significant difference was found between the glutamine treatment and saline (Figure 4-10). The 1.0 mmol/kg

A



B

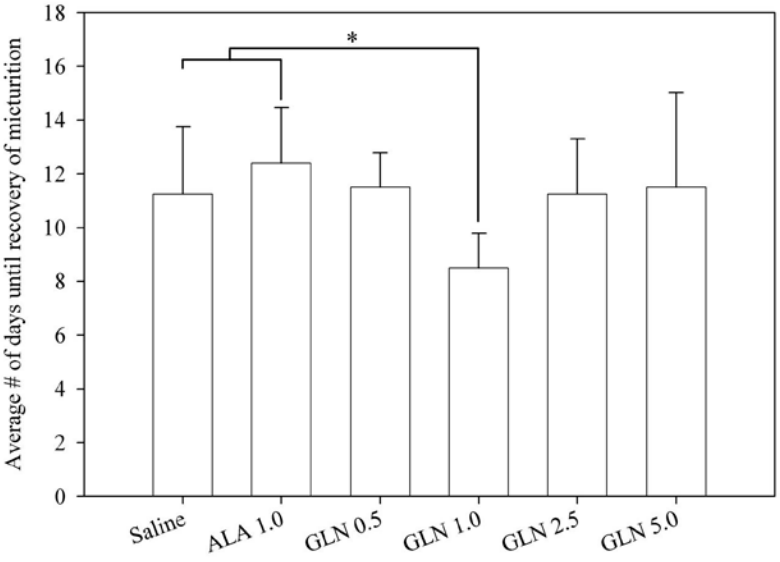


Figure 4-10: Micturition recovers more rapidly with glutamine treatment than saline. Micturition was assessed by quantifying the days post-surgery until the bladder size was first assessed as small (A) or recovery of micturition (B). Values (ie GLN 0.5) indicate dose in mmol/kg. * denotes a significant difference ($P < 0.05$). Data are presented as mean ± standard deviation. ALA - alanine, GLN - glutamine

glutamine concentration significantly lowered the number of days until a small bladder was seen compared to the saline treated (5.75 ± 0.96 vs 9.25 ± 2.63, P = 0.022). Similarly, it was the 1.0 mmol/kg glutamine concentration that had the most potent effect on rate of micturition recovery. This concentration significantly decreased the amount of time to recovery of micturition (8.52 ± 1.29) compared to both saline (11.25 ± 2.50, P = 0.015) and 1.0 mmol/kg alanine treatments (12.40 ± 2.07, P = 0.013). Higher doses of glutamine did not significantly improve bladder recovery function.

4.3.2.2 Locomotory recovery

While a dose of 1 mmol/kg significantly improved BBB scores compared to saline vehicle and alanine controls, doses of 0.5 and 5 mmol glutamine kg did not. Only the 1 mmol/kg glutamine significantly increased BBB scores above that of saline and alanine which are both significantly different from all other experimental groups (Figure 4-11). The 2.5 mmol/kg concentration was significantly different from the 5.0 mmol/kg group (11.6 ± 0.7 vs 8.2 ± 1.1, P = 0.004). All six glutamine treated animals in both the 1.0 and 2.5 mmol/kg groups achieved a BBB score of 9 or better, while all other groups had only 2 or 3. As would be expected, the same 1.0 mmol/kg dose that was most potent in improving BBB scores had a similar effect on the inclined plane scores. This dose of glutamine resulted in significantly higher inclined plane scores compared to both the saline control (45.0 ± 2.0 vs 27.5 ± 6.2, P = 0.038) and the 5.0 mmol/kg group (25.0 ± 3.5, P = 0.029) (Figure 4-12).

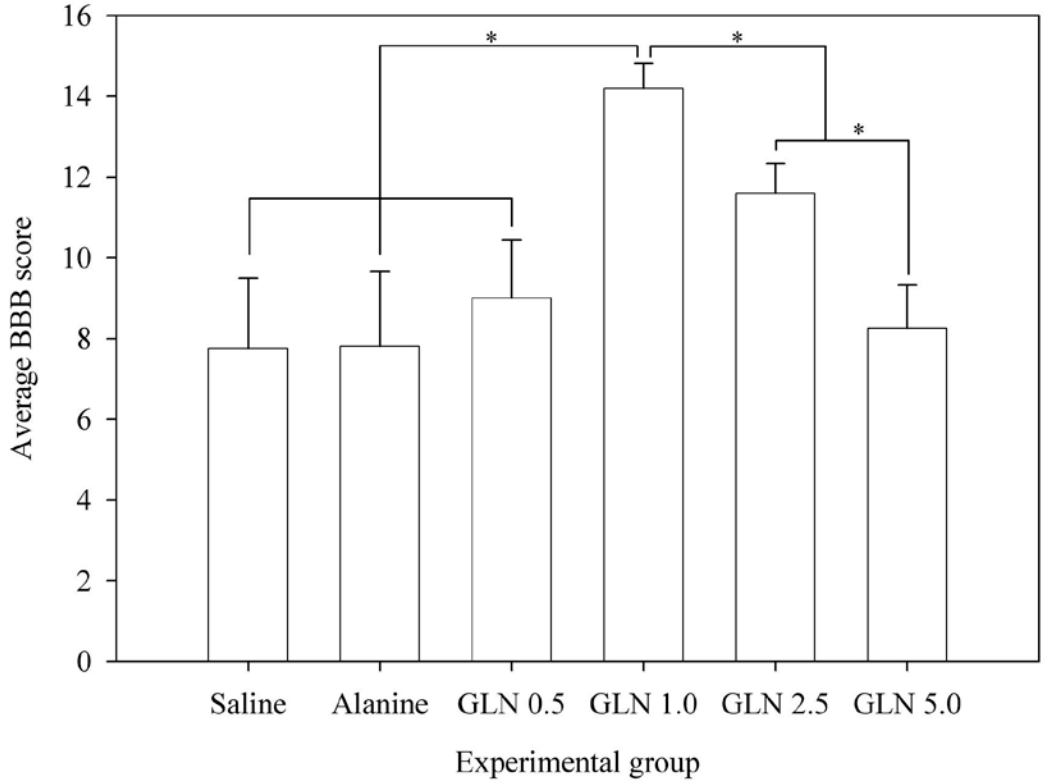


Figure 4-11: BBB scores are increased significantly with glutamine treatment in a concentration dependant fashion. Data shown are the measurements taken 6 weeks after injury and are expressed as mean ± standard error. * denotes significant difference ($P < 0.05$). GLN - glutamine

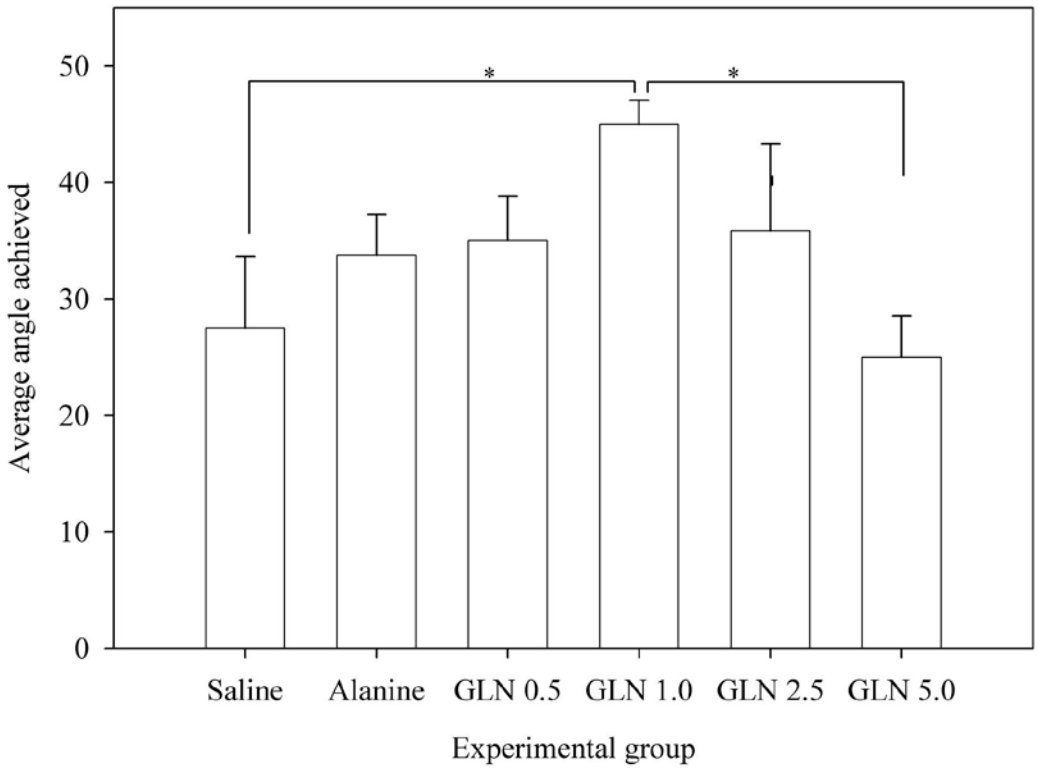


Figure 4-12: Glutamine treatment increases inclined plane scores in a concentration dependent fashion. * denotes significant difference ($P < 0.05$). Data shown are the measurements taken 6 weeks after injury and are expressed as mean ± standard error. GLN - glutamine

4.3.2.3 Weight changes six weeks after surgery

Over the six week span of the study the amount of weight that animals gained significantly differed amongst experimental groups (Figure 4-13). Sham animals treated with saline gained an average of 139.3 ± 14.8 g, while glutamine treated (5 mmol/kg) shams gained only 116.4 ± 21.9 g. This was a surprising finding, however it did not reach statistical significance (P = 0.264). The saline treated sham animals gained significantly more weight than three of the injured groups namely: saline, alanine and 5.0 mmol/kg glutamine. Of the injured animals, those gaining the most weight were in the 1.0 mmol/kg group (110.0 ± 12.4 g) which was significantly higher than both the saline (62.5 ± 7.7 g, P = 0.006) and the 5.0 mmol/kg glutamine group (69.0 ± 8.5 g, P = 0.035).

4.3.2.4 Histological analysis

No detectable changes were seen in the histology of animals undergoing the sham (laminectomy surgery) and receiving either saline or glutamine (Data not shown). Glutamine dose-dependant changes in histology were apparent particularly between the 0.5 and 1.0 mmol/kg concentrations (Figure 4-14 A-F). The histology samples from the 0.5 mmol/kg treatment group appear very similar to that of the saline and alanine treated samples. Many of these samples have basic tissues organization and do not contain distinct areas of LFB positive tissue and dorsal horns of gray matter. In comparison, all other concentrations of glutamine had distinct regions of LFB positive tissues (particularly in areas of the RST and sometimes CST) and contained viable tissues within the dorsal horns. Similar to that discussed previously, doses that were associated with improved

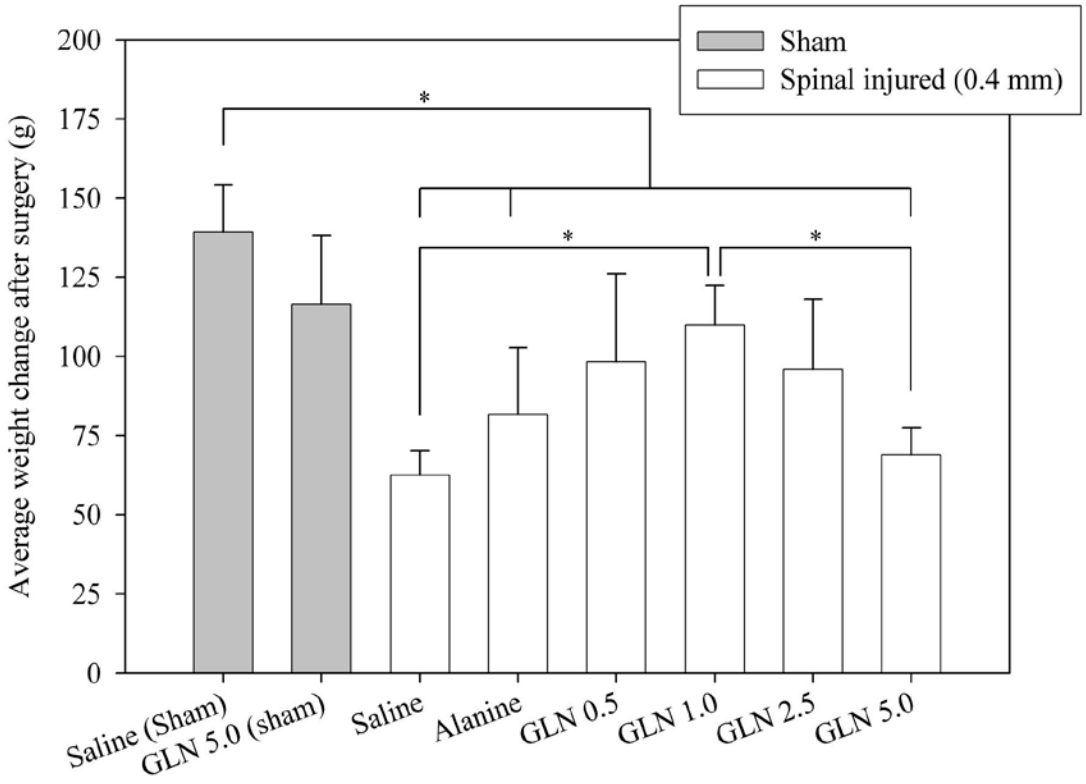


Figure 4-13: Glutamine treatment significantly increased the amount of weight gain in the six week post-surgical period. Measurements were calculated as the difference between the pre-surgical and 6 week weights. * denotes a significant difference ($P < 0.05$). Data are expressed as mean ± standard deviation. GLN - glutamine

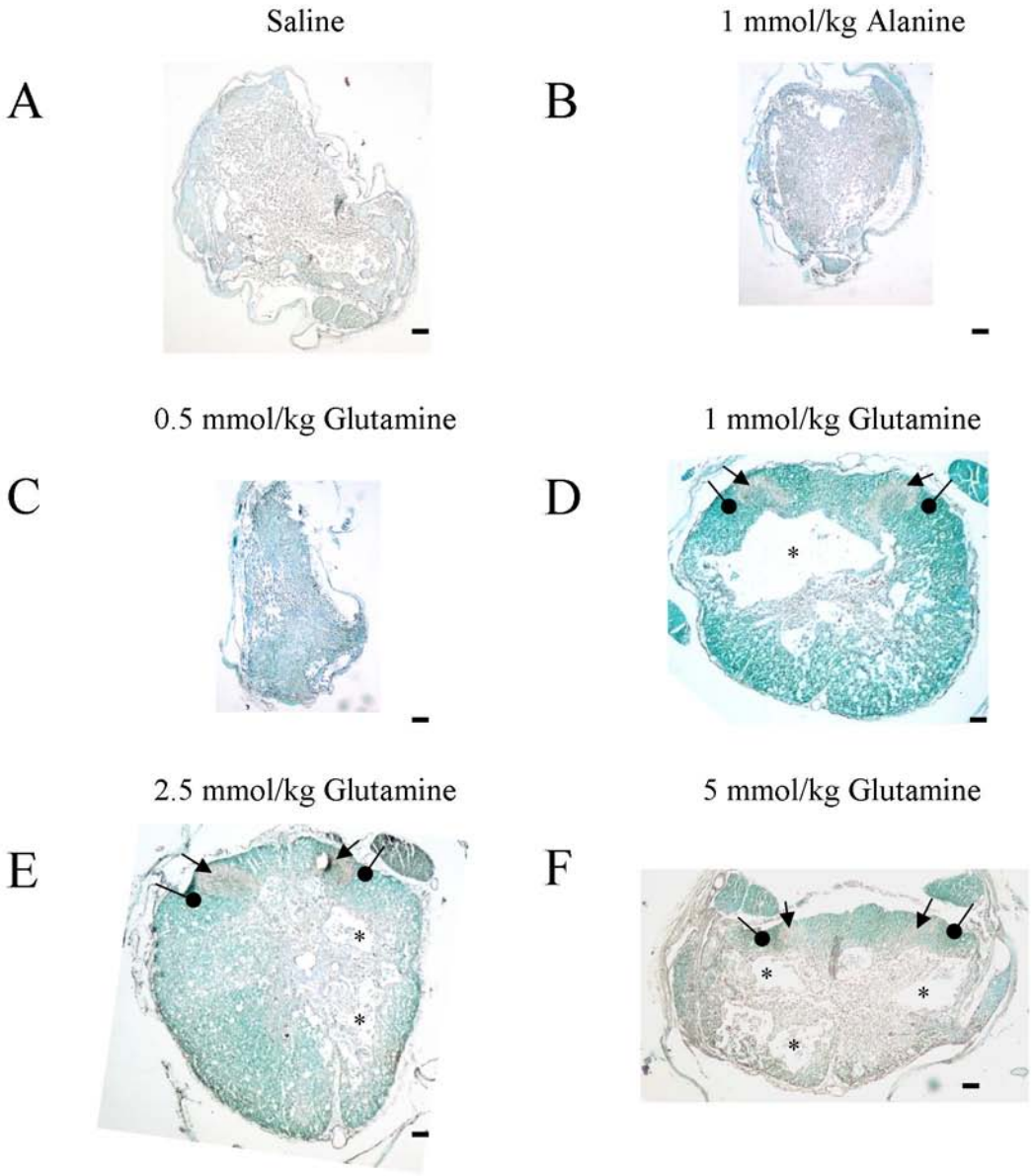


Figure 4-14: Glutamine treatment improved maintenance of tissue morphology 6 weeks after 0.4 mm injury. Sections are representative through the epicentre of injury * denotes areas of cystic cavities, black arrows indicate areas of spared white matter, round-ended arrows indicate areas of the rubrospinal tract that are spared, Scale bar represents 100 µm.

locomotor function (1.0 mmol/kg) still had cystic cavities, however they appeared to have less debris within them. Saline and alanine tissues had cystic cavities filled with debris which, on closer examination, contained many cells with macrophage-type morphology. When comparing tissues of injured animals treated with 1 or 5 mmol glutamine/kg, animals treated with 5 mmol/kg the tissues have less area positively stained with luxol fast blue and appear to have increased astrocyte scarring (Figure 4-14 F).

Quantification of white matter spared correlated well with the locomotor recovery trends. The maximum amount of white matter spared was 8.27 ± 0.39 % in the 1.0 mmol/kg group which was not different from the 2.5 mmol/kg group (7.93 ± 0.44 %) (Figure 4-15). These two groups had significantly more white matter sparing than the saline and alanine controls as well as the 0.5 mmol/kg glutamine treatment.

4.4 Discussion

4.4.1 Biochemical changes

Following SCI there is an increase in oxidative stress (Kamencic et al., 2001; Aksenova et al., 2002; Bao and Liu, 2002; Xu et al., 2005) and a body wide inflammatory response which has been demonstrated not only to damage the spinal cord (Popovich et al., 1999; Beattie, 2004; Fleming et al., 2006; Keane et al., 2006; Trivedi et al., 2006) but also the kidneys and lungs (Gris et al., 2008). In our model, SCI induced oxidative stress as measured by a decrease in whole blood GSH, was prevented by glutamine treatment. This finding is similar to that seen previously using the modified

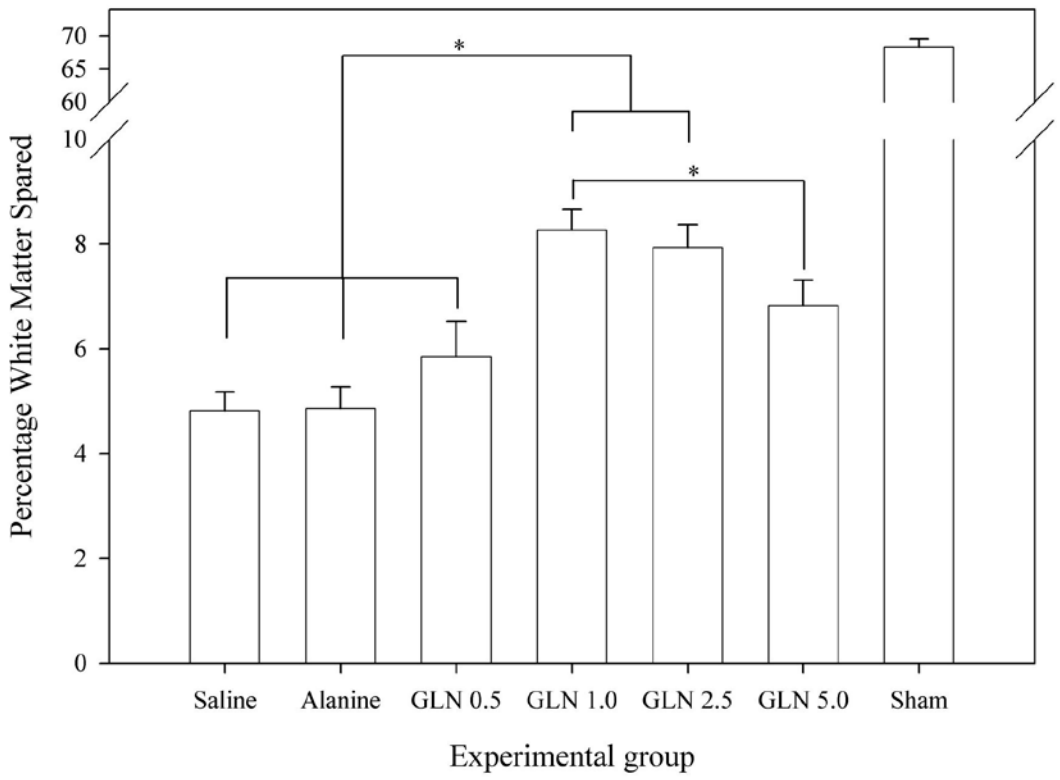


Figure 4-15: Glutamine increased white matter sparing at the site of injury . Measurements are calculated as the number of stained pixels in the injured tissues and expressed as a percentage of those in the sham. Data shown are the measurements taken 6 weeks after injury and are expressed as mean ± standard error. * denotes significant difference ($P < 0.05$). GLN - glutamine

aneurysm clip model which showed improvements in blood and spinal cord GSH concentrations following glutamine treatment at the same 1 mmol/kg concentration. Our study is in agreement with other research demonstrating the ability of glutamine to help maintain GSH concentrations after various injuries in numerous tissues (Hong et al., 1992; Harward et al., 1994; Denno et al., 1996).

The most likely mechanism for a glutamine-mediated increase in GSH concentration is the improved maintenance of intracellular glutamate concentrations which, in addition to being a constituent of GSH (Kvamme et al., 2001), will alleviate the GSH induced inhibition of GCL (Meister, 1988) (Figure 4-1). One major contributor to cellular damage following neurotrauma is excitotoxicity caused by an excess amount of glutamate in the extracellular space (Perez Velazquez et al., 1997). As we have demonstrated previously glutamine will increase GSH in injured spinal tissue thus improving the ability to scavenge ROS. Reactive oxygen species inhibit glutamate transporters which are responsible for regulating extracellular concentrations of glutamate by astrocytes (Rao et al., 2003). By increasing uptake of glutamate, GSH synthesis should be increased through previously described mechanisms and excitotoxicity would be inhibited by decreasing the concentration of extracellular glutamate.

It has been previously demonstrated that maintenance of GSH in the spinal cord strongly correlated with decreased lipid peroxidation (Lucas et al., 2002), improved locomotor recovery and increased spinal tissue spared (Kamencic et al., 2001). Within the brain, GSH has been demonstrated to improve maintenance of blood brain barrier (Agarwal and Shukla, 1999). Similarly, GSH ablation has been shown to significantly

increase loss of tissue, motor function and increased signs of lipid peroxidation and pro-apoptotic factors (Genovese et al., 2007).

Additional mechanisms which should be considered are that of the role of glutamine in energy production in the form of ATP and glucose. As a precursor for glutamate and therefore α-ketoglutarate, glutamine can be shunted into the Kreb's cycle as an intermediate leading to ATP production (Peng et al., 1993). This is supported by the finding that glutamine treatment increased ATP production in cardiomyocytes following induced myocardial infarction in rats (Kumar and Anandan, 2007). Secondly, it is estimated that glutamine can contribute up to 8-10% of glucose formation in healthy human (Nurjhan et al., 1995) and is thought to significantly increase in states of stress. Dogs, in a state of chronic acidosis, generate 40% of their plasma glucose through renal gluconeogenesis which relies heavily on glutamine (Pitts et al., 1972).

4.4.2 Recovery of micturition

The focus of SCI research is beginning to shift to better reflect the priorities of spinal trauma patients which include bladder, bowel and sexual function even above that of locomotor ability in the paraplegic population (Anderson, 2004). Therefore, it is important to incorporate measurements, such as this admittedly crude assessment of micturition, into an experiment. These measurements require no additional equipment, are easily integrated into the experimental design and provide potentially important insight into the relationship between injury, therapeutics and micturition. Measurements showed good reproducibility between experiments and enabled us to assess the therapeutic potential of glutamine with regards to the rate of recovery of micturition.

The glutamine-mediated improvement in the rate of recovery of micturition most likely reflects the improved maintenance of white matter across the injury site rather than a direct effect on the micturition pathways. Glutamine treated tissues and those of lesser injury severity (0.8 and 1.0 mm) appeared to have more sparing in the lateral white matter than the more severe and saline treated tissues.

Partial recovery of micturition spontaneously after injury has been documented previously and has been linked to segmental reflexes (Basso et al., 1995; Ko et al., 1999). Although under normal circumstances the pontine micturition pathways are required for micturition many changes occur within the spinal cord after injury which facilitate the recovery of partial function. Normally the Aδ fibers would relay information regarding the level of distention in the bladder to the pons and the C-fibers which carry temperature and pain information have very little involvement in micturition (de Groat, 1995; de Groat and Yoshimura, 2006). Following SCI however this changes and the sprouting of C-fibers partially replace the spinobulbospinal pathway with a sacral reflex (de Groat et al., 1990). The establishment of this new reflex does not lead to recovery of control of micturition, but rather can lead to bladder hyperactivity and decreased micturition threshold (Chancellor and de Groat, 1999).

Recovery of coordination, which requires supraspinal input occurs over a longer period of time and is thought to involve the maintenance of bulbospinal projections (Pikov et al., 1998). The white matter spared within the injury site may contain descending tracts responsible for micturation. These tracts, which carry information from the pontine micturition center, are believed to travel in the lateral white matter in both cats (McMahon et al., 1982) and humans (Nathan and Smith, 1958). Similar to that seen previously (Kloos et al., 2005), white matter sparing appeared first in the dorsal and

ventral white matter and only in the glutamine treated or less severely injured was white matter spared in the lateral portions of the cord spared.

4.4.3. Recovery of Motor Function

Recovery of locomotor function was both injury and concentration dependent. The significant improvement in BBB score seen in the 0.4 mm injury with glutamine treatment plateaued with no further improvements seen in the less severe injuries. It is possible that although 1.0 mmol/kg is the optimal concentration for the 0.4 mm injury, as seen in the second experiment, an alternate concentration for the lesser injuries would have been more potent. Additionally, this could simply be the maximal response possible for glutamine treatment alone.

Surprisingly, the highest BBB scores from injured animals did not correspond to the highest measurement of white matter sparing in the injured animals. The 8.27 % in the 0.4 mm glutamine treated resulted in a BBB score of 13.7 where the glutamine treated 1.0 mm had 10.36 % white matter and a BBB score of 12.8. The 2.1 % difference in total white matter spared corresponded to a 0.9 point decrease in BBB. White matter sparing occurs in a non-linear fashion as demonstrated by Kloos et al. (2005) who showed that an increase of white matter from 6.4 to 7.5 % (1.1 % change in total white matter) increases BBB scores from 9.6 to 11.3 (1.7 point change), whereas to increase the scores a further 2.6 points would require and additional 10% of the white matter to be spared. There is also clearly variability within the correlation which seems more prominent as you move between BBB scores of 10 and 15. Kloos found no difference in BBB scores between two injuries (0.5 and 0.7 mm) regardless of a ~ 8-10 % difference in white matter sparing. An important point is non-linearity of BBB scale itself as a one

point change can sometimes indicate improvements in gross motor movements which may require fewer white matter tracts and others indicate a fine motor improvement which would require a much greater amount of white matter sparing.

The efficacy of glutamine, when compared to other therapeutic strategies, is consistently equal or more potent. The most commonly used treatment for SCI clinically is MPO which is currently under great debate (Hurlbert, 2000; Hurlbert and Moulton, 2002; Sayer et al., 2006). When studied in the rat, MPO had no effect on BBB scores or white matter sparing following a contusive injury (Takami et al., 2002; Weaver et al., 2005). These findings are in contrast to a more recent study using a dorsal hemi-section model which demonstrated a 3 point increase in BBB with MPO alone and a 4 point improvement when combined with a soluble nogo - 66 receptor (Ji et al., 2005).

There are many other studies using antioxidant/anti-inflammatory strategies as treatments including omega -3 fatty acids which demonstrate a 3 point BBB increase (~8.5 to ~11.5) one week after dorsal hemisection. Resveratrol, which has been shown to have antioxidant and anti-inflammatory effects, showed minimal improvement both with mobility and inclined plane scores after contusion injury (Ates et al., 2006). Even, working on a strategy similar to our own to increase GSH, a cell permeable form of GSH, GSH monoethyl ester, improved BBB scores only ~ 2 points (9 to 11) after contusion injury. Lastly, a nitroxide antioxidant, Tempol, was assessed using a contusion model and found improved BBB scores (2.7 points) (Hillard et al., 2004). Even the anti-apoptotic soluable Fas receptor treatment was only able to improve BBB scores by ~ 2.5 points and did not improve inclined plane scores (Ackery et al., 2006).

To our knowledge, the only compounds which compare to the potency of glutamine (increasing BBB scores by 6 points, 8 to 14) and work through antioxidant

and/or anti-inflammatory pathways are two that were previously examined in our lab, a pro-cysteine compound L-2-oxothizolidine-4-carboxylate (OTZ) and the polyphenolic quercetin. Following a 50 g aneurysm clip compression injury, OTZ decreased oxidative stress and improved BBB scores from 6 to 13 (Kamencic et al., 2001) and the polyphenolic quercetin increased BBB scores from 8 to ~14 (Schultke et al., 2003).

4.4.4. Rate of weight gain

As expected, the 1.0 mmol/kg glutamine treatment resulted in an increased rate of weight gain following injury compared with saline treatment. We have previously demonstrated that glutamine treatment helped to maintain muscle mass and function after SCI (Golding et al., 2006b) most likely due to a combination of sustained innervation and decreased proteolysis (Blomqvist et al., 1995). Surprisingly, the 5 mmol/kg treatment did not improve the rate of weight gain in these animals. Although there could be many reasons for this, one fact to consider in analyzing this is the lack of an increase in white matter sparing at the site of injury with this dose. Therefore, we could speculate that 5 mmol/kg is outside of the therapeutic dosing window for this compound. Perhaps at five times the optimal concentration, although the additional glutamine may be having beneficial effects in other tissues, there may be a partial enhancement of the excitotoxic mechanisms within the spinal cord. As we have seen in previous experiments, 5 mmol/kg glutamine does not improve spinal cord GSH concentrations which we believe is a key mechanism in effect for glutamine treatment.

4.4.5. Histological changes after injury

Histology from these experiments demonstrated classic characteristics of moderate to severe spinal cord injuries, namely cavitation, loss of most gray matter, loss of white matter corresponding to injury severity, increased cellularity, and glial scar formation (Behrmann et al., 1992; Basso et al., 1996; Gruner et al., 1996; Kloos et al., 2005). Optimal glutamine treatment, as seen in the 0.4 mm injury and 1.0 mmol/kg concentration demonstrated significant increases in white matter sparing in dorsal, ventral and lateral funiculi. Saline and alanine treated tissues had classic signs of SCI including cystic cavities and the formation of scar tissue. Similar to the previous experiment, the tissues from animals treated with glutamine 1.0 mmol/kg differed in that they had less scar tissue and a lower degree of cellularity. These findings appear on the surface to be signs of decreased inflammation within the spinal cord. We believe that there are two main mechanisms through which glutamine may have induced this effect.

Glutamine has been demonstrated to be essential as an energy source for many immune cells (Parry-Billings et al., 1990; Ogle et al., 1994; Newsholme and Calder, 1997; Kew et al., 1999; Pithon-Curi et al., 2002b; Pithon-Curi et al., 2002a; Pithon-Curi et al., 2004; Peng et al., 2006a) and deficiencies in glutamine have been linked to immunosuppression (Parry-Billings et al., 1990; Oehler et al., 2002; Rogeri and Rosa, 2005). By maintaining or increasing plasma glutamine concentrations immune function can be maintained, however we were uncertain as to the impact this aspect would have on recovery. Research examining the importance of the immune response to both destructive and reparative processes has been the subject of many studies and reviews (Popovich et al., 1999; Bethea and Dietrich, 2002; Popovich et al., 2002; Profyris et al.,

2004; Jones et al., 2005; Trivedi et al., 2006; Donnelly and Popovich, 2008; Popovich and Longbrake, 2008).

Although the initial inflammatory response has been shown to be detrimental due to an overabundance of reactive oxygen species (Fleming et al., 2006; Trivedi et al., 2006), the clearance of cellular debris to allow for regrowth is essential to the repair process (Schwartz and Yoles, 2006; Ziv et al., 2006). Glutamine can decrease the negative effects of the initial inflammatory response by increasing the concentration of GSH both at the site of injury and globally. Secondly, by maintaining plasma concentrations of glutamine, immune function is maintained to allow for clean up of the injury site to promote regrowth. In this way it is plausible that glutamine treatment was able to optimize the inflammatory response following SCI.

As discussed previously, in the lesser injured tissues, and with many of those treated with glutamine, the lateral white matter was spared which is believed to contain the descending tracts from the pontine micturition center. Appearance of the lateral white matter correlates with improved locomotor function and micturition. The preferential loss of lateral white matter compared to dorsal or ventral in these animals is consistent with previous findings (Kloos et al., 2005). Sparing in the dorsal white matter in areas of the CST and RST corresponded to animals achieving higher BBB scores. Kloos et al. (2005) also demonstrated the correlation between the percentage of white matter spared and the subsequent locomotor recovery, as shown previously, our values for of BBB and white matter correspond well to that of Kloos et al. (2005).

The majority of gray matter was lost regardless of injury severity or treatment, the exception being dorsal horn gray matter which was seen more commonly in the glutamine treated animals and in those undergoing less severe injuries. The more

superficial portions of the horn were seen, predominantly at the levels of lamina I and II which correspond to areas associated with nociception (Iggo et al., 1985; Steedman et al., 1985). Although not directly tested in these animals, there was no apparently neuropathic pain below the site of injury in any animals in these experiments which we believe can be explained through the following three mechanisms.

First, the appearance of neuropathic pain following SCI in rats has also been associated with a greater than 10% white matter sparing (Kloos et al., 2005). In these experiments very few animals that had > 10% white matter sparing had received the 1.0 mmol/kg glutamine treatment. Secondly, many studies have found a link between inflammation and induction of neuropathic pain (Tal, 1999; Watkins et al., 2003; Tegeder et al., 2004; Peng et al., 2006b; Kawasaki et al., 2008; Kleibeuker et al., 2008). As described above, glutamine treatment by decreasing oxidative stress ought to decrease inflammation and therefore may inhibit neuropathic pain through this mechanism as well. Lastly, although GABA is initially raised following trauma (Diaz-Ruiz et al., 2007), it decreases long term (Vaiva et al., 2006). Activation of GABA receptors have been demonstrated repeatedly to have anti-nociceptive effects (Patel et al., 2001; Franek et al., 2004; Gwak et al., 2006) and, as glutamine can act as a precursor for GABA (Peng et al., 1993) it is possible that our treatment prevents or decreases neuropathic pain by maintaining or increasing concentrations of GABA.

4.4.6 Conclusions

We have demonstrated the potency of glutamine treatment in both the aneurysm clip and the modified forceps model of SCI. Glutamine substantially improved motor function and tissues sparing in addition to improving the rate of recovery of micturition

and weight gain post-surgery. Although our research focused on the use of glutamine to increase GSH content, we acknowledge the many mechanisms through which glutamine my affect SCI recovery, some of which were previously discussed in this paper.

Glutamine is currently widely used in clinical trials including low-weight newborns (Parimi et al., 2005; Parimi and Kalhan, 2007), burn victims (Parry-Billings et al., 1990; Gore and Jahoor, 1994; Ogle et al., 1994; Garrel et al., 2003; Peng et al., 2004), surgical patients (Wilmore, 2001), multiple trauma patients (Houdijk et al., 1998; Houdijk and van Leeuwen, 2000; Bakalar et al., 2006), cancer patients (Klimberg and McClellan, 1996; Yoshida et al., 2001; Savarese et al., 2003) and even neurotrauma (Richards et al., 2003; Falcao de Arruda and de Aguilar-Nascimento, 2004; Ronne Engstrom et al., 2005; Berg et al., 2006). To our knowledge, despite the ever growing literature on glutamine clinically, there are no studies demonstrating a negative side effect of glutamine supplementation in physiologically relevant concentrations. In contrast, there are an ever growing number of manuscripts detailing the benefits of glutamine supplementation in a wide variety of pathological situation as recently reviewed (Wischmeyer, 2008).

One of the concerns regarding the use of glutamine as a therapeutic in cases of neurotrauma is the possibility that increased availability of glutamine will increase extracellular glutamate concentrations and lead to an exacerbation of excitotoxicity. Recently, glutamine was given intravenously to head trauma patients and concentrations of glutamate in the CSF were monitored and found to be unchanged (Ronne Engstrom et al., 2005). Ronne Engstrom et al. infused 10 g of glutamine over 5 hours resulting in a dose of approximately 0.14 g/kg/day (assuming an average weight of 70 kg). A second study in head trauma patients, administered 0.34 g/kg glutamine over 20 hours and also

found no significant increases in glutamate concentrations in CSF samples (Berg et al., 2006). Comparatively, our studies administered glutamine in two injections 12 hours apart at an optimal dose of 1 mmol/kg (0.14 g/kg), for an overall dose of 0.28 g/kg/day. Therefore, the range of glutamine doses (0.5 - 10 mmol/kg/injection or 0.14 - 2.8 g/kg/day) used in these experiments are clinically relevant and our optimal dose (0.28 g/kg/day) is similar to those demonstrated to be safe in neurotrauma patients (Ronne Engstrom et al., 2005; Berg et al., 2006). Since glutamine supplementation has shown such promise clinically and has had potent therapeutic effects in spinal injured rats, we suggest that human clinical trials should be initiated to determine whether glutamine supplementation would also have therapeutic effects in the acutely spinal injured human.

5.0 CHAPTER

DISCUSSION

5.1 Summary of results

Each of the hypotheses examined were upheld through the course of these experiments. Our first hypothesis stated that the administration of glutamine will improve maintenance of GSH levels after SCI. This hypothesis was upheld valid in both spinal cord tissue and blood in both SCI models as seen in sections 2.4.1 and 4.3.1.1. The second hypothesis of this project stated that better maintaining GSH levels following injury will improve tissue sparing and motor recovery. This hypothesis was upheld both in the aneurysm clip model of spinal injury as well as in the forceps model of spinal injury (Section 2.4.2.1 & 2.4.2.2) and again using the modified forceps model (Sections 4.3.1.2 – 4.3.1.4, 4.3.2.1 – 4.3.1.2). Lastly, our final hypothesis stated that glutamine treatment efficacy will be reproducible across spinal injury models. The substantial improvements seen in all aspects of recovery in both SCI models demonstrate that the final hypothesis has validity (Chapters 2 & 3).

5.1.1 Glutamine increased GSH

The preliminary experiment performed in astrocyte cultures demonstrated the important role for glutamine in governing GSH synthesis. Our *in vivo* experiments demonstrated a similar finding. Using the modified aneurysm clip we found that both spinal and blood GSH could be increased with glutamine supplementation. The clinical efficacy of glutamine treatment had a very narrow window as only the 1 mmol/kg dose increased GSH in the spinal cord at the site of injury. Additionally, the highest

concentration used (10 mmol/kg) showed detrimental effects in decreasing spinal GSH beyond that of the injury itself. Glutamine treatment increased blood GSH concentrations after SCI using both the aneurysm clip and modified forceps model thus demonstrating the ability of glutamine to decrease body wide oxidative stress. Experiments using the modified forceps demonstrated the dependence of the effect on injury severity showing that although glutamine increased GSH concentration in all other injuries, in the most severe injury (0.2 mm) glutamine was not able to increase GSH.

5.1.2 Bladder function

Using the modified forceps model a significant correlation was found between injury severity and the rate of micturition recovery. Most likely due to improved maintenance of white matter tracts in the spinal cord, glutamine treatment significantly increased the rate of micturition recovery. The effect of glutamine was dose-dependent with the most effective dose being 1.0 mmol/kg which was also the most effective in all other measurements.

5.1.3 Recovery of Motor Function

Glutamine treatment improved locomotor function in both the aneurysm clip and modified forceps models. Although the aneurysm clip induced a more severe injury than even the most severe injury in the forceps model (0.2mm), the efficacy of glutamine treatment was similar showing a 7 and 5 point improvements on BBB scale respectively. This finding was surprising as the most severe injury using the forceps model which induced less tissue disruption in the spinal cord than the aneurysm clip, showed no

improvement with glutamine which may be a result of the differences in specific mechanisms of injury between models.

While developing the forceps compression model we were able to demonstrate a significant correlation between the injury severity and both the open field (BBB) and inclined plane scores of the saline treated animals. These strong correlations in addition to the similarities between our results and those of Gruner et al. (1996) demonstrated the consistency of the model.

5.1.4 Histology and white matter sparing

The histology of the saline treated animals seen in both the aneurysm clip and forceps models was very similar to that previously described in the literature. Spinal cord tissues from our injured animals demonstrated cystic cavities, infiltration of immune cells, glial scarring and peripheral white matter sparing. In the majority of saline or alanine treated tissues, the gray matter was destroyed while in the glutamine treated tissues there was a larger amount of gray matter was seen, predominantly in the dorsal horns.

Quantification of white matter area confirmed what we could see qualitatively, a strong correlation between white matter sparing and injury severity using the forceps model. The significant correlations of white matter sparing to both injury severity and BBB scores gave us strong evidence of the consistency and accuracy of the new model. Our correlations for white matter and BBB scores corresponded well to that of previous works in both the aneurysm clip and forceps models. As expected, given the significant increases in locomotion, glutamine treatment resulted in a dose-dependent increase in

the amount of white matter spared at the site of injury compared to saline treated animals.

5.2 Future aims

5.2.1 Laboratory research: experimental design

The route of administration of glutamine for this study was by IP injection. It would be important, given the small range in therapeutic dose, to complete a small study using both oral and intravenous administration of glutamine to determine if the change in bioavailability between routes would alter the most effective dose following SCI. Glutamine clinically is given by both enteral and parenteral routes, therefore determining the effect of administration route on therapeutic dose will be important for future clinical trials.

I would recommend the administration of glutamine at first contact with a potential SCI patient. Given that glutamine has been demonstrated not just to be safe, but to be beneficial in wide variety of pathologies including multiple trauma (Houdijk et al., 1998) and as most SCI patients have multiple other injuries (Burney et al., 1993) I feel that this would be a safe broad spectrum therapeutic. Additionally, given the delay between the onset of injury and treatment clinically, the efficacy of a delayed glutamine treatment should be assessed to better mimic the clinical setting for SCI patients and determine if alterations in the recommended dose are necessary.

5.2.2 Laboratory research: oxidative stress

My research has been able to demonstrate the therapeutic potential of glutamine in the field of SCI. Glutamine has the ability to improve the maintenance of GSH not

only in the spinal cord at the site of injury, but systemically as seen in whole blood samples. While the central effect increases tissue sparing with other associated positive findings, the systemic effect may be equally important as it should decrease the oxidative damage and associated inflammation to other tissues seen after SCI, including the lungs and kidneys (Gris et al., 2008). Therefore the examination of organs, in addition to the spinal cord should be carried out to determine the full therapeutic effect of glutamine following SCI.

Although this project has used glutamine to augment the production of GSH, other compounds, such as OTZ have been equally successful (Kamencic et al., 2001). It would be interesting to co-administer glutamine and OTZ as these compounds work independently on the GSH synthesis pathways and the combination may further increase GSH synthesis and thereby improve recovery. Another compound that may be useful to combine with glutamine and/or OTZ is the polyphenolic quercetin which has both anti-apoptotic and anti-inflammatory properties.

5.2.3 Laboratory experiments: alternate mechanisms of action

Given the multitude of possible mechanisms of action for glutamine, it would be helpful to elucidate the primary functions. ^{13}C glutamine studies using nuclear magnetic resonance (NMR) would be useful in identifying the most important metabolites following SCI and glutamine treatment. This study would further increase our understanding of the altered metabolic needs of the SCI patient in addition to improving our understanding of how glutamine exerts its effects.

Beyond the function of glutamine as an antioxidant precursor, it is important to better understand the key mechanisms through which this treatment results in such

potent neuroprotection. Of particular interest would be the relationship of glutamine to the maintenance of the immune system function. As discussed previously, the immune response following SCI has both positive and negative consequences. Glutamine administration acts to maintain immune system function (Newsholme, 2001) in addition to decreasing the oxidative stress, therefore determining the role of glutamine separately in the early and late phases of the immune response would be beneficial. Although our administration of glutamine one hour following injury had significant positive effects, this may be enhanced if an anti-inflammatory was also given during the early inflammatory phase.

Glutamine treatment has been shown to maintain muscle GSH and decrease muscle proteolysis following injury (Blomqvist et al., 1995; Flaring et al., 2003). In collaboration with Dr. Benjamin Rosser's lab, I have also demonstrated the ability of glutamine treatment to decrease muscle atrophy and partially prevent changes in muscle cell characteristics after injury (Golding et al., 2006a). Whether this is an effect of better sustained innervation of the muscle or decreased proteolysis due to the glutamine supplementation is uncertain and should be examined further.

Given the effect of glutamine on the rate of micturition recovery, it would be useful to examine if this finding was purely due to increased maintenance of white matter tracts within the spinal cord or from additional mechanisms. The significant role of glutamine in maintenance of the acid base balance and the production of urea may be mechanisms through which glutamine can influence urine production and whether this could influence micturition.

Although glutamine improved the sparing of dorsal horn gray matter which is associated with nociception, no obvious neuropathic was seen in our animals. Glutamine

administration has been demonstrated to increase GABA (Peng et al., 1993) which is associated with anti-nociceptive effects (Patel et al., 2001; Franek et al., 2004; Gwak et al., 2006) and has been shown to decrease following trauma (Vaiva et al., 2006). Through this mechanism, glutamine may be able to modulate allodynia following SCI and thus is an additional pathway which should be examined.

5.2.4 Clinical trials

Despite the numerous clinical trials involving glutamine in various pathologic paradigms there are no studies using glutamine as a therapeutic for SCI. One reason for this is the concern over the possible enhancement of excitotoxicity following glutamine administration due to the potential increase in production of glutamate. Two different clinical studies have demonstrated that glutamine treatment would increase plasma glutamine concentrations but not that of cerebral glutamate in neurointensive care patients (Engstrom et al., 2005; Berg et al., 2006). Additionally, glutamine has been used in combination with probiotics in head trauma patients with findings of decreased rate of infections and decrease length of stay in intensive care (Falcao de Arruda and de Aguilar-Nascimento, 2004). These findings demonstrate the safety of glutamine clinically in situations of neurotrauma and lend support for the use of glutamine as a treatment for SCI.

As most SCI patients have multiple injuries (Burney et al., 1993) and as glutamine has been proven effective in treating a wide variety of injuries with no apparent detrimental side effects, we believe that a clinical trial using glutamine is warranted. The use of glutamine as a therapeutic has the advantages of having multifaceted mechanisms of action, proven clinical safety in neurotrauma/neurosurgery

patients and is inexpensive and readily available. Lastly, unlike many of the manufactured drugs, the effect of glutamine on multiple organ systems, in both normal and pathologic situations, has been extensively studied clinically and therefore relatively well understood. With the breadth of knowledge regarding glutamine clinically, I believe that the potential for translation of the potent effect of glutamine as a SCI treatment from the laboratoryto the clinic is significant.

5.3 Significance and conclusions

The standard clinical care for SCI involves stabilization of the vertebral column, maintenance of arterial blood pressure and the administration of the steroid MPO. Currently, the use of MPO is under great debate due to the inconsistent findings of positive results in humans (Bracken et al., 1984; Bracken et al., 1990; Nesathurai, 1998; Hurlbert, 2000; Hurlbert and Moulton, 2002; Sayer et al., 2006) and in rats (Liu and McAdoo, 1993; Behrmann et al., 1994; Takami et al., 2002; Ates et al., 2006) and the significant negative side effects (Bracken et al., 1984; Bracken et al., 1997).

Despite the enormous effort, both financially and intellectually, being put towards the goal of an effective SCI treatment, no new clinical treatments have come on the market recently. One significant challenge in studying potential SCI therapeutics is the difference in the effect of the drug between rodents and humans. Many compounds are studied extensively in rats and mice prior to an examination in humans, which although it is ethically sound, results in many years of study for a compound that may be ineffective in the human animal. The use of glutamine in this project had the benefit of having been extensively studied in humans prior to our use of it as an SCI treatment.

Glutamine has been the subject of many studies, both clinical and laboratory based, for decades which have been recently reviewed (Lacey and Wilmore, 1990; Newsholme and Calder, 1997; Neu et al., 2002; Roth et al., 2002; Oehler and Roth, 2003; Savarese et al., 2003; Melis et al., 2004; Wischmeyer, 2006, 2008). The interest in glutamine stems from the many tissues and systems that it plays a role in either directly or as a metabolic precursor for another active compound. To my knowledge there are no clinical studies demonstrating a negative side effect of glutamine treatment regardless of the numerous paradigms in which it was tested, including in clinical neurotrauma (Falcao de Arruda and de Aguilar-Nascimento, 2004; Ronne Engstrom et al., 2005; Berg et al., 2006).

The efficacy of glutamine treatment as a therapeutic for SCI exceeded my expectations. By supplementing the body with an amino acid shown to decrease to 54% of basal plasma levels in patients following SCI (Rogeri and Rosa, 2005), I was able to enhance or maintain the body's own reparatory mechanisms. The simplicity of using an amino acid as a treatment is contrasted by the diverse pathways through which glutamine may exert its therapeutic effects. My project focused on the improved maintenance of GSH as the primary mechanism of action; however we do recognize the many additional mechanisms which may have contributed.

In our experiments glutamine has demonstrated many effects, including: 1) increasing GSH concentrations within the spinal cord at the site of injury, 2) increasing blood GSH in two separate injury models, 3) improving white matter sparing at the site of injury, 4) improving rate of recovery of micturition in an injury and dose dependent manner and 5) improving locomotor function as indicated by improved open field and inclined plane scores.

In addition to the above findings, we were able to contribute to the known characteristics of glutamine as a treatment. The safety of glutamine treatment in SCI was examined by completing dose response studies which demonstrated that only at 10 times the therapeutic dose are negative effects, presumably due to excitotoxicity, seen. I also assessed the efficacy of glutamine as a treatment in two separate injury models and across six different injury severities. Given the efficacy, safety and the breadth of knowledge regarding the use of glutamine clinically, I feel that glutamine would be a safe and effective treatment option for SCI patients and see no need to delay the initiation of a clinical trial.

Chapter 6.0

Bibliography

Ackery A, Robins S, Fehlings MG (2006) Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury. J Neurotrauma 23:604-616.

Agarwal R, Shukla G (1999) Potential role of cerebral glutathione in the maintenance of blood-brain barrier integrity in rat. Neurochem Res 24:1507-1514.

Aksenova M, Butterfield DA, Zhang SX, Underwood M, Geddes JW (2002) Increased protein oxidation and decreased creatine kinase BB expression and activity after spinal cord contusion injury. J Neurotrauma 19:491-502.

Anderson DK (1992) Chemical and cellular mediators in spinal cord injury. J Neurotrauma 9:143-145; discussion 145-146.

Anderson KD (2004) Targeting recovery: priorities of the spinal cord-injured population. J Neurotrauma 21:1371-1383.

Asensi M, Sastre J, Pallardo FV, Lloret A, Lehner M, Garcia-de-la Asuncion J, Vina J (1999) Ratio of reduced to oxidized glutathione as indicator of oxidative stress status and DNA damage. Methods Enzymol 299:267-276.

Ates O, Cayli S, Altinoz E, Gurses I, Yucel N, Kocak A, Yologlu S, Turkoz Y (2006) Effects of resveratrol and methylprednisolone on biochemical, neurobehavioral and histopathological recovery after experimental spinal cord injury. Acta Pharmacol Sin 27:1317-1325.

Austgen TR, Chen MK, Dudrick PS, Copeland EM, Souba WW (1992) Cytokine regulation of intestinal glutamine utilization. Am J Surg 163:174-179; discussion 179-180.

Bakalar B, Duska F, Pachl J, Fric M, Otahal M, Pazout J, Andel M (2006) Parenterally administered dipeptide alanyl-glutamine prevents worsening of insulin sensitivity in multiple-trauma patients. Crit Care Med 34:381-386.

Baldwin SA, Broderick R, Osbourne D, Waeg G, Blades DA, Scheff SW (1998) The presence of 4-hydroxynonenal/protein complex as an indicator of oxidative stress after experimental spinal cord contusion in a rat model. J Neurosurg 88:874-883.

Banati RB, Graeber MB (1994) Surveillance, intervention and cytotoxicity: is there a protective role of microglia? Dev Neurosci 16:114-127.

Bao F, Liu D (2002) Peroxynitrite generated in the rat spinal cord induces neuron death and neurological deficits. Neuroscience 115:839-849.

Baptiste DC, Fehlings MG (2006) Pharmacological approaches to repair the injured spinal cord. J Neurotrauma 23:318-334.

Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12:1-21.

Basso DM, Beattie MS, Bresnahan JC (1996) Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Exp Neurol 139:244-256.

Beattie MS (2004) Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. Trends Mol Med 10:580-583.

Behar KL, Rothman DL (2001) In vivo nuclear magnetic resonance studies of glutamate-gamma-aminobutyric acid-glutamine cycling in rodent and human cortex: the central role of glutamine. J Nutr 131:2498S-2504S; discussion 2523S-2494S.

Behrmann DL, Bresnahan JC, Beattie MS (1994) Modeling of acute spinal cord injury in the rat: neuroprotection and enhanced recovery with methylprednisolone, U-74006F and YM-14673. Exp Neurol 126:61-75.

Behrmann DL, Bresnahan JC, Beattie MS, Shah BR (1992) Spinal cord injury produced by consistent mechanical displacement of the cord in rats: behavioral and histologic analysis. J Neurotrauma 9:197-217.

Berg A, Bellander BM, Wanecek M, Gamrin L, Elving A, Rooyackers O, Ungerstedt U, Wernerman J (2006) Intravenous glutamine supplementation to head trauma patients leaves cerebral glutamate concentration unaffected. Intensive Care Med 32:1741-1746.

Beril Gok H, Solaroglu I, Okutan O, Cimen B, Kaptanoglu E, Palaoglu S (2007) Metoprolol treatment decreases tissue myeloperoxidase activity after spinal cord injury in rats. J Clin Neurosci 14:138-142.

Bethea JR (2000) Spinal cord injury-induced inflammation: a dual-edged sword. Prog Brain Res 128:33-42.

Bethea JR, Dietrich WD (2002) Targeting the host inflammatory response in traumatic spinal cord injury. Curr Opin Neurol 15:355-360.

Bistrian B (2004) Practical recommendations for immune-enhancing diets. J Nutr 134:2868S-2872S; discussion 2895S.

Blight AR (1991) Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology. J Neurol Sci 103:156-171.

Blomqvist BI, Hammarqvist F, von der Decken A, Wernerman J (1995) Glutamine and alpha-ketoglutarate prevent the decrease in muscle free glutamine concentration

and influence protein synthesis after total hip replacement. Metabolism 44:1215-1222.

Bracken MB, Collins WF, Freeman DF, Shepard MJ, Wagner FW, Silten RM, Hellenbrand KG, Ransohoff J, Hunt WE, Perot PL, Jr., et al. (1984) Efficacy of methylprednisolone in acute spinal cord injury. Jama 251:45-52.

Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon J, et al. (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. N Engl J Med 322:1405-1411.

Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, Fehlings M, Herr DL, Hitchon PW, Marshall LF, Nockels RP, Pascale V, Perot PL, Jr., Piepmeier J, Sonntag VK, Wagner F, Wilberger JE, Winn HR, Young W (1997) Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. Jama 277:1597-1604.

Brand K, Leibold W, Luppa P, Schoerner C, Schulz A (1986) Metabolic alterations associated with proliferation of mitogen-activated lymphocytes and of lymphoblastoid cell lines: evaluation of glucose and glutamine metabolism. Immunobiology 173:23-34.

Brown SA, Goringe A, Fegan C, Davies SV, Giddings J, Whittaker JA, Burnett AK, Poynton CH (1998) Parenteral glutamine protects hepatic function during bone marrow transplantation. Bone Marrow Transplant 22:281-284.

Buchman AL (2001) Glutamine: commercially essential or conditionally essential? A critical appraisal of the human data. Am J Clin Nutr 74:25-32.

Burney RE, Maio RF, Maynard F, Karunas R (1993) Incidence, characteristics, and outcome of spinal cord injury at trauma centers in North America. Arch Surg 128:596-599.

Burrin D, Davis T (2004) Proteins and amino acids in enteral nutrition. Curr Opin Clin Nutr Metab Care 7:79-87.

Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L (1998) Acute inflammatory response in spinal cord following impact injury. Exp Neurol 151:77-88.

Chan PH (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 21:2-14.

Chancellor MB, de Groat WC (1999) Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder. J Urol 162:3-11.

Chang WK, Yang KD, Chuang H, Jan JT, Shaio MF (2002) Glutamine protects activated human T cells from apoptosis by up- regulating glutathione and Bcl-2 levels. Clin Immunol 104:151-160.

Chatzipanteli K, Garcia R, Marcillo AE, Loor KE, Kraydieh S, Dietrich WD (2002) Temporal and segmental distribution of constitutive and inducible nitric oxide synthases after traumatic spinal cord injury: effect of aminoguanidine treatment. J Neurotrauma 19:639-651.

Christen Y (2000) Oxidative stress and Alzheimer disease. Am J Clin Nutr 71:621S-629S.

Christman JW, Lancaster LH, Blackwell TS (1998) Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. Intensive Care Med 24:1131-1138.

Coghlin Dickson TM, Wong RM, offrin RS, Shizuru JA, Johnston LJ, Hu WW, Blume KG, Stockerl-Goldstein KE (2000) Effect of oral glutamine supplementation during bone marrow transplantation. JPEN J Parenter Enteral Nutr 24:61-66.

Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr 15:133-159.

da Gama Torres HO, Vilela EG, da Cunha AS, Goulart EM, Souza MH, Aguirre AC, Azevedo WM, Lodi FM, Silva AA, Bittencourt HN (2008) Efficacy of glutamine-supplemented parenteral nutrition on short-term survival following Allo-SCT: a randomized study. Bone Marrow Transplant.

Damian AC, Pitts RF (1970) Rates of glutaminase I and glutamine synthetase reactions in rat kidney in vivo. Am J Physiol 218:1249-1255.

Daniel WW (1987) Biostatistics: A Foundation for Analysis in the Health Sciences, 4th Edition: John Wiley & Sons, Inc.

Darmaun D, Matthews DE, Bier DM (1986) Glutamine and glutamate kinetics in humans. Am J Physiol 251:E117-126.

de Barros Filho TE, Molina AE (2008) Analysis of the sensitivity and reproducibility of the Basso, Beattie, Bresnahan (BBB) scale in Wistar rats. Clinics 63:103-108.

de Groat WC (1995) Mechanisms underlying the recovery of lower urinary tract function following spinal cord injury. Paraplegia 33:493-505.

de Groat WC, Yoshimura N (2006) Mechanisms underlying the recovery of lower urinary tract function following spinal cord injury. Prog Brain Res 152:59-84.

de Groat WC, Kawatani M, Hisamitsu T, Cheng CL, Ma CP, Thor K, Steers W, Roppolo JR (1990) Mechanisms underlying the recovery of urinary bladder function following spinal cord injury. J Auton Nerv Syst 30 Suppl:S71-77.

Dechelotte P, Hasselmann M, Cynober L, Allaouchiche B, Coeffier M, Hecketsweiler B, Merle V, Mazerolles M, Samba D, Guillou YM, Petit J, Mansoor O, Colas G, Cohendy R, Barnoud D, Czernichow P, Bleichner G (2006) L-alanyl-L-glutamine dipeptide-supplemented total parenteral nutrition reduces infectious complications and glucose intolerance in critically ill patients: the French controlled, randomized, double-blind, multicenter study. Crit Care Med 34:598-604.

Denno R, Rounds JD, Faris R, Holejko LB, Wilmore DW (1996) Glutamine-enriched total parenteral nutrition enhances plasma glutathione in the resting state. J Surg Res 61:35-38.

DeVivo MJ (1997) Causes and costs of spinal cord injury in the United States. Spinal Cord 35:809-813.

Di Giovanni S, Knoblach SM, Brandoli C, Aden SA, Hoffman EP, Faden AI (2003) Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. Ann Neurol 53:454-468.

Diaz-Ruiz A, Salgado-Ceballos H, Montes S, Maldonado V, Tristan L, Alcaraz-Zubeldia M, Rios C (2007) Acute alterations of glutamate, glutamine, GABA, and other amino acids after spinal cord contusion in rats. Neurochem Res 32:57-63.

Ditor DS, John SM, Roy J, Marx JC, Kittmer C, Weaver LC (2007) Effects of polyethylene glycol and magnesium sulfate administration on clinically relevant neurological outcomes after spinal cord injury in the rat. J Neurosci Res 85:1458-1467.

Dohrmann GJ, Allen WE (1975) Microcirculation of traumatized spinal cord. A correlation of microangiography and blood flow patterns in transitory and permanent paraplegia. J Trauma 15:1003-1013.

Donnelly DJ, Popovich PG (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378-388.

Engel JM, Muhling J, Weiss S, Lohr T, Simonis Y, Menges T, Hempelmann G (2003) Low plasma glutamine after multiple trauma: relationship with intracellular glutamine in polymorphonuclear neutrophils during prolonged ICU stay. Acta Anaesthesiol Scand 47:707-713.

Engesser-Cesar C, Anderson AJ, Basso DM, Edgerton VR, Cotman CW (2005) Voluntary wheel running improves recovery from a moderate spinal cord injury. J Neurotrauma 22:157-171.

Engstrom ER, Hillered L, Enblad P, Karlsson T (2005) Cerebral interstitial levels of glutamate and glutamine after intravenous administration of nutritional amino acids in neurointensive care patients. Neurosci Lett 384:7-10.

Fairholm D, Turnbull I (1970) Microangiographic study of experimental spinal injuries in dogs and rabbits. Surg Forum 21:453-455.

Falcao de Arruda IS, de Aguilar-Nascimento JE (2004) Benefits of early enteral nutrition with glutamine and probiotics in brain injury patients. Clin Sci (Lond) 106:287-292.

Fehlings MG, Tator CH (1995) The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. Exp Neurol 132:220-228.

Fehlings MG, Tator CH, Linden RD (1989) The effect of nimodipine and dextran on axonal function and blood flow following experimental spinal cord injury. J Neurosurg 71:403-416.

Flaring UB, Rooyackers OE, Wernerman J, Hammarqvist F (2003) Glutamine attenuates post-traumatic glutathione depletion in human muscle. Clin Sci (Lond) 104:275-282.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, Pasquale-Styles M, Dietrich WD, Weaver LC (2006) The cellular inflammatory response in human spinal cords after injury. Brain 129:3249-3269.

Flohe L (1978) Glutathione peroxidase: fact and fiction. Ciba Found Symp:95-122.

Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L (1997) Redox regulation of NF-kappa B activation. Free Radic Biol Med 22:1115-1126.

Franek M, Vaculin S, Rokyta R (2004) GABA(B) receptor agonist baclofen has non-specific antinociceptive effect in the model of peripheral neuropathy in the rat. Physiol Res 53:351-355.

Frayn KN, Khan K, Coppack SW, Elia M (1991) Amino acid metabolism in human subcutaneous adipose tissue in vivo. Clin Sci (Lond) 80:471-474.

Garcia-de-Lorenzo A, Zarazaga A, Garcia-Luna PP, Gonzalez-Huix F, Lopez-Martinez J, Mijan A, Quecedo L, Casimiro C, Usan L, del Llano J (2003) Clinical evidence for enteral nutritional support with glutamine: a systematic review. Nutrition 19:805-811.

Garrel D, Patenaude J, Nedelec B, Samson L, Dorais J, Champoux J, D'Elia M, Bernier J (2003) Decreased mortality and infectious morbidity in adult burn patients given enteral glutamine supplements: a prospective, controlled, randomized clinical trial. Crit Care Med 31:2444-2449.

Gebhardt R, Mecke D (1983) Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. Embo J 2:567-570.

Genovese T, Mazzon E, Esposito E, Muia C, Di Paola R, Di Bella P, Bramanti P, Cuzzocrea S (2007) Role of endogenous glutathione in the secondary damage in experimental spinal cord injury in mice. Neurosci Lett 423:41-46.

Gilgun-Sherki Y, Rosenbaum Z, Melamed E, Offen D (2002) Antioxidant therapy in acute central nervous system injury: current state. Pharmacol Rev 54:271-284.

Golding JD, MacDonald ST, Juurlink BH, Rosser BW (2006a) The effect of glutamine on locomotor performance and skeletal muscle myosins following spinal cord injury in rats. J Appl Physiol 101:1045-1052.

Golding JD, Rigley Macdonald ST, Juurlink BH, Rosser B (2006b) The effect of glutamine on locomotor performance and skeletal muscle myosins following spinal cord injury in rats. J Appl Physiol 101:1045-1052.

Gore DC, Jahoor F (1994) Glutamine kinetics in burn patients. Comparison with hormonally induced stress in volunteers. Arch Surg 129:1318-1323.

Gras G, Porcheray F, Samah B, Leone C (2006) The glutamate-glutamine cycle as an inducible, protective face of macrophage activation. J Leukoc Biol 80:1067-1075.

Grill R, Murai K, Blesch A, Gage FH, Tuszynski MH (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. J Neurosci 17:5560-5572.

Grimble RF (2001) Nutritional modulation of immune function. Proc Nutr Soc 60:389-397.

Gris D, Hamilton EF, Weaver LC (2008) The systemic inflammatory response after spinal cord injury damages lungs and kidneys. Exp Neurol 211:259-270.

Gruner J, Yee A, Blight A (1996) Histological and functional evaluation of experimental spinal cord injury: evidence of a stepwise response to graded compression. Brain Res 729:90-101.

Gruner JA (1992) A monitored contusion model of spinal cord injury in the rat. J Neurotrauma 9:123-126; discussion 126-128.

Gstraunthaler G, Holcomb T, Feifel E, Liu W, Spitaler N, Curthoys NP (2000) Differential expression and acid-base regulation of glutaminase mRNAs in gluconeogenic LLC-PK(1)-FBPase(+) cells. Am J Physiol Renal Physiol 278:F227-237.

Gulick AM, Fahl WE (1995) Mammalian glutathione S-transferase: regulation of an enzyme system to achieve chemotherapeutic efficacy. Pharmacol Ther 66:237-257.

Gwak YS, Tan HY, Nam TS, Paik KS, Hulsebosch CE, Leem JW (2006) Activation of spinal GABA receptors attenuates chronic central neuropathic pain after spinal cord injury. J Neurotrauma 23:1111-1124.

Hagg T, Oudega M (2006) Degenerative and spontaneous regenerative processes after spinal cord injury. J Neurotrauma 23:264-280.

Hall ED, Yonkers PA, Horan KL, Braughler JM (1989) Correlation between attenuation of posttraumatic spinal cord ischemia and preservation of tissue vitamin E by the 21-aminosteroid U74006F: evidence for an in vivo antioxidant mechanism. J Neurotrauma 6:169-176.

Hall JC, Dobb G, Hall J, de Sousa R, Brennan L, McCauley R (2003) A prospective randomized trial of enteral glutamine in critical illness. Intensive Care Med 29:1710-1716.

Halperin ML, Bun-Chen C (1987) Plasma glutamine and renal ammoniagenesis in dogs with chronic metabolic acidosis. Am J Physiol 252:F474-479.

Hammarqvist F, Westman B, Leijonmarck CE, Andersson K, Wernerman J (1996) Decrease in muscle glutamine, ribosomes, and the nitrogen losses are similar after laparoscopic compared with open cholecystectomy during the immediate postoperative period. Surgery 119:417-423.

Harlan JM (1987) Consequences of leukocyte-vessel wall interactions in inflammatory and immune reactions. Semin Thromb Hemost 13:434-444.

Harrison KA, Murphy RC (1995) Isoleukotrienes are biologically active free radical products of lipid peroxidation. J Biol Chem 270:17273-17278.

Harward T, Coe D, Souba W, Klingman N, Seeger J (1994) Glutamine preserves gut glutathione levels during intestinal ischemia/reperfusion. J Surg Res 56:351-355.

Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN (1987) Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am J Physiol 253:H699-703.

Hillard VH, Peng H, Zhang Y, Das K, Murali R, Etlinger JD, Zeman RJ (2004) Tempol, a nitroxide antioxidant, improves locomotor and histological outcomes after spinal cord contusion in rats. J Neurotrauma 21:1405-1414.

Hong R, Rounds J, Helton W, Robinson M, Wilmore D (1992) Glutamine preserves liver glutathione after lethal hepatic injury. Ann Surg 215:114-119.

Houdijk AP, van Leeuwen PA (2000) Glutamine-enriched enteral nutrition in multiple trauma patients. Nutrition 16:70-71.

Houdijk AP, Rijnsburger ER, Jansen J, Wesdorp RI, Weiss JK, McCamish MA, Teerlink T, Meuwissen SG, Haarman HJ, Thijs LG, van Leeuwen PA (1998) Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. Lancet 352:772-776.

Hundal HS, Rennie MJ, Watt PW (1987) Characteristics of L-glutamine transport in perfused rat skeletal muscle. J Physiol 393:283-305.

Hurlbert RJ (2000) Methylprednisolone for acute spinal cord injury: an inappropriate standard of care. J Neurosurg 93:1-7.

Hurlbert RJ (2006) Strategies of medical intervention in the management of acute spinal cord injury. Spine 31:S16-21; discussion S36.

Hurlbert RJ, Moulton R (2002) Why do you prescribe methylprednisolone for acute spinal cord injury? A Canadian perspective and a position statement. Can J Neurol Sci 29:236-239.

Iggo A, Steedman WM, Fleetwood-Walker S (1985) Spinal processing: anatomy and physiology of spinal nociceptive mechanisms. Philos Trans R Soc Lond B Biol Sci 308:235-252.

Iwasa K, Ikata T, Fukuzawa K (1989) Protective effect of vitamin E on spinal cord injury by compression and concurrent lipid peroxidation. Free Radic Biol Med 6:599-606.

Ji B, Li M, Budel S, Pepinsky RB, Walus L, Engber TM, Strittmatter SM, Relton JK (2005) Effect of combined treatment with methylprednisolone and soluble Nogo-66 receptor after rat spinal cord injury. Eur J Neurosci 22:587-594.

Jones TB, McDaniel EE, Popovich PG (2005) Inflammatory-mediated injury and repair in the traumatically injured spinal cord. Curr Pharm Des 11:1223-1236.

Joshi M, Fehlings MG (2002) Development and characterization of a novel, graded model of clip compressive spinal cord injury in the mouse: Part 1. Clip design, behavioral outcomes, and histopathology. J Neurotrauma 19:175-190.

Juurlink (1999) Management of Oxidative Stress in the CNS: The Many Roles of Glutathione. Neurotox Res 1:119-140.

Juurlink BH, Paterson PG (1998) Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. J Spinal Cord Med 21:309-334.

Juurlink BH, Thorburne SK, Hertz L (1998) Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. Glia 22:371-378.

Kakulas BA (1987) The clinical neuropathology of spinal cord injury. A guide to the future. Paraplegia 25:212-216.

Kamencic H, Lyon A, Paterson PG, Juurlink BH (2000) Monochlorobimane fluorometric method to measure tissue glutathione. Anal Biochem 286:35-37.

Kamencic H, Griebel RW, Lyon AW, Paterson PG, Juurlink BH (2001) Promoting glutathione synthesis after spinal cord trauma decreases secondary damage and promotes retention of function. Faseb J 15:243-250.

Kaptanoglu E, Sen S, Beskonakli E, Surucu HS, Tuncel M, Kilinc K, Taskin Y (2002) Antioxidant actions and early ultrastructural findings of thiopental and propofol in experimental spinal cord injury. J Neurosurg Anesthesiol 14:114-122.

Katoh D, Ikata T, Katoh S, Hamada Y, Fukuzawa K (1996) Effect of dietary vitamin C on compression injury of the spinal cord in a rat mutant unable to synthesize ascorbic acid and its correlation with that of vitamin E. Spinal Cord 34:234-238.

Katrusiak AE, Paterson PG, Kamencic H, Shoker A, Lyon AW (2001) Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl-glycine, homocysteine and glutathione in plasma and cell extracts. J Chromatogr B Biomed Sci Appl 758:207-212.

Kawasaki Y, Zhang L, Cheng JK, Ji RR (2008) Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J Neurosci 28:5189-5194.

Kayali H, Ozdag MF, Kahraman S, Aydin A, Gonul E, Sayal A, Odabasi Z, Timurkaynak E (2005) The antioxidant effect of beta-Glucan on oxidative stress status in experimental spinal cord injury in rats. Neurosurg Rev 28:298-302.

Keane RW, Davis AR, Dietrich WD (2006) Inflammatory and apoptotic signaling after spinal cord injury. J Neurotrauma 23:335-344.

Keller JN, Mark RJ, Bruce AJ, Blanc E, Rothstein JD, Uchida K, Waeg G, Mattson MP (1997) 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. Neuroscience 80:685-696.

Kew S, Wells SM, Yaqoob P, Wallace FA, Miles EA, Calder PC (1999) Dietary glutamine enhances murine T-lymphocyte responsiveness. J Nutr 129:1524-1531.

Khan M, Griebel R (1983a) Acute Spinal Cord Injury in the Rat: Comparison of Three Experimental Techniques. Can J Neurol Sci 10:161-165.

Khan M, Griebel R (1983b) Acute spinal cord injury in the rat: comparison of three experimental techniques. Can J Neurol Sci 10:161-165.

Khogali SE, Harper AA, Lyall JA, Rennie MJ (1998) Effects of L-glutamine on post-ischaemic cardiac function: protection and rescue. J Mol Cell Cardiol 30:819-827.

Kilberg MS, Handlogten ME, Christensen HN (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J Biol Chem 255:4011-4019.

Kleibeuker W, Gabay E, Kavelaars A, Zijlstra J, Wolf G, Ziv N, Yirmiya R, Shavit Y, Tal M, Heijnen CJ (2008) IL-1 beta signaling is required for mechanical allodynia induced by nerve injury and for the ensuing reduction in spinal cord neuronal GRK2. Brain Behav Immun 22:200-208.

Klimberg VS, McClellan JL (1996) Claude H. Organ, Jr. Honorary Lectureship. Glutamine, cancer, and its therapy. Am J Surg 172:418-424.

Kloos A, Fisher L, Detloff M, Hassenzahl D, Basso D (2005) Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. Exp Neurol 191:251-265.

Ko HY, Ditunno JF, Jr., Graziani V, Little JW (1999) The pattern of reflex recovery during spinal shock. Spinal Cord 37:402-409.

Kobrine AI, Evans DE, Rizzoli HV (1979) Experimental acute balloon compression of the spinal cord. Factors affecting disappearance and return of the spinal evoked response. J Neurosurg 51:841-845.

Krebs HA (1935) Metabolism of amino-acids: The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. Biochem J 29:1951-1969.

Kuhn KS, Schuhmann K, Stehle P, Darmaun D, Furst P (1999) Determination of glutamine in muscle protein facilitates accurate assessment of proteolysis and de novo synthesis-derived endogenous glutamine production. Am J Clin Nutr 70:484-489.

Kumar S, Kumar R, Sharma SB, Jain BK (2007) Effect of oral glutamine administration on oxidative stress, morbidity and mortality in critically ill surgical patients. Indian J Gastroenterol 26:70-73.

Kumar SHS, Anandan R (2007) Biochemical studies on the cardioprotective effect of glutamine on tissue antioxidant defense system in isoprenaline-induced myocardial infarction in rats. J Clin Biochem Nutr 40:49-55.

Kvamme E, Torgner IA, Roberg B (2001) Kinetics and localization of brain phosphate activated glutaminase. J Neurosci Res 66:951-958.

Kwon BK, Tetzlaff W, Grauer JN, Beiner J, Vaccaro AR (2004) Pathophysiology and pharmacologic treatment of acute spinal cord injury. Spine J 4:451-464.

Lacey JM, Wilmore DW (1990) Is glutamine a conditionally essential amino acid? Nutr Rev 48:297-309.

Li H, Marshall ZM, Whorton AR (1999) Stimulation of cystine uptake by nitric oxide: regulation of endothelial cell glutathione levels. Am J Physiol 276:C803-811.

Li Y, Field PM, Raisman G (1997) Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. Science 277:2000-2002.

Liu D, McAdoo D (1993) Methylprednisolone reduces excitatory amino acid release following experimental spinal cord injury. Brain Res 609:293-297.

Liu D, Ling X, Wen J, Liu J (2000) The role of reactive nitrogen species in secondary spinal cord injury: formation of nitric oxide, peroxynitrite, and nitrated protein. J Neurochem 75:2144-2154.

Liu TZ, Stern A, Morrow JD (1998) The isoprostanes: unique bioactive products of lipid peroxidation. An overview. J Biomed Sci 5:415-420.

Lovat R, Preiser JC (2003) Antioxidant therapy in intensive care. Curr Opin Crit Care 9:266-270.

Lucas JH, Wheeler DG, Guan Z, Suntres Z, Stokes BT (2002) Effect of glutathione augmentation on lipid peroxidation after spinal cord injury. J Neurotrauma 19:763-775.

Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP (1997) A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. J Neurochem 68:255-264.

Matthews JC, Anderson KJ (2002) Recent advances in amino acid transporters and excitatory amino acid receptors. Curr Opin Clin Nutr Metab Care 5:77-84.

McBean GJ, Flynn J (2001) Molecular mechanisms of cystine transport. Biochem Soc Trans 29:717-722.

McMahon SB, Morrison JF, Spillane K (1982) An electrophysiological study of somatic and visceral convergence in the reflex control of the external sphincters. J Physiol 328:379-387.

Meister A (1974) Glutathione, metabolism and function via the gamma-glutamyl cycle. Life Sci 15:177-190.

Meister A (1988) Glutathione metabolism and its selective modification. J Biol Chem 263:17205-17208.

Melis GC, ter Wengel N, Boelens PG, van Leeuwen PA (2004) Glutamine: recent developments in research on the clinical significance of glutamine. Curr Opin Clin Nutr Metab Care 7:59-70.

Nakanishi T, Sugawara M, Huang W, Martindale RG, Leibach FH, Ganapathy ME, Prasad PD, Ganapathy V (2001) Structure, function, and tissue expression pattern of human SN2, a subtype of the amino acid transport system N. Biochem Biophys Res Commun 281:1343-1348.

Nathan PW, Smith MC (1958) The centrifugal pathway for micturition within the spinal cord. J Neurol Neurosurg Psychiatry 21:177-189.

Nesathurai S (1998) Steroids and spinal cord injury: revisiting the NASCIS 2 and NASCIS 3 trials. J Trauma 45:1088-1093.

Neu J, DeMarco V, Li N (2002) Glutamine: clinical applications and mechanisms of action. Curr Opin Clin Nutr Metab Care 5:69-75.

Neumann H, Wekerle H (1998) Neuronal control of the immune response in the central nervous system: linking brain immunity to neurodegeneration. J Neuropathol Exp Neurol 57:1-9.

Newsholme EA, Calder PC (1997) The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition 13:728-730.

Newsholme P (2001) Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? J Nutr 131:2515S-2522S; discussion 2523S-2514S.

Newsholme P, Procopio J, Lima MM, Pithon-Curi TC, Curi R (2003a) Glutamine and glutamate-their central role in cell metabolism and function. Cell Biochem Funct 21:1-9.

Newsholme P, Lima MM, Procopio J, Pithon-Curi TC, Doi SQ, Bazotte RB, Curi R (2003b) Glutamine and glutamate as vital metabolites. Braz J Med Biol Res 36:153-163.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.

Norenberg MD, Smith J, Marcillo A (2004) The pathology of human spinal cord injury: defining the problems. J Neurotrauma 21:429-440.

NSCISC (2006) SPINAL CORD INJURY Facts and Figures at a Glance. National Spinal Cord Injury Statistical Center.

Nurjhan N, Bucci A, Perriello G, Stumvoll M, Dailey G, Bier DM, Toft I, Jenssen TG, Gerich JE (1995) Glutamine: a major gluconeogenic precursor and vehicle for interorgan carbon transport in man. J Clin Invest 95:272-277.

Oehler R, Roth E (2003) Regulative capacity of glutamine. Curr Opin Clin Nutr Metab Care 6:277-282.

Oehler R, Pusch E, Dungel P, Zellner M, Eliasen MM, Brabec M, Roth E (2002) Glutamine depletion impairs cellular stress response in human leucocytes. Br J Nutr 87 Suppl 1:S17-21.

Ogle C, Ogle J, Mao J, Simon J, Noel J, Li B, Alexander J (1994) Effect of glutamine on phagocytosis and bacterial killing by normal and pediatric burn patient neutrophils. JPEN J Parenter Enteral Nutr 18:128-133.

Owen OE, Felig P, Morgan AP, Wahren J, Cahill GF, Jr. (1969) Liver and kidney metabolism during prolonged starvation. J Clin Invest 48:574-583.

Parimi PS, Kalhan SC (2007) Glutamine supplementation in the newborn infant. Semin Fetal Neonatal Med 12:19-25.

Parimi PS, Kadrofske MM, Gruca LL, Hanson RW, Kalhan SC (2005) Amino acids, glutamine, and protein metabolism in very low birth weight infants. Pediatr Res 58:1259-1264.

Park E, Velumian AA, Fehlings MG (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J Neurotrauma 21:754-774.

Parry-Billings M, Evans J, Calder PC, Newsholme EA (1990) Does glutamine contribute to immunosuppression after major burns? Lancet 336:523-525.

Patel S, Naeem S, Kesingland A, Froestl W, Capogna M, Urban L, Fox A (2001) The effects of GABA(B) agonists and gabapentin on mechanical hyperalgesia in models of neuropathic and inflammatory pain in the rat. Pain 90:217-226.

Peng L, Hertz L, Huang R, Sonnewald U, Petersen SB, Westergaard N, Larsson O, Schousboe A (1993) Utilization of glutamine and of TCA cycle constituents as precursors for transmitter glutamate and GABA. Dev Neurosci 15:367-377.

Peng X, Yan H, You Z, Wang P, Wang S (2004) Effects of enteral supplementation with glutamine granules on intestinal mucosal barrier function in severe burned patients. Burns 30:135-139.

Peng X, Yan H, You Z, Wang P, Wang S (2006a) Glutamine granule-supplemented enteral nutrition maintains immunological function in severely burned patients. Burns 32:589-593.

Peng XM, Zhou ZG, Glorioso JC, Fink DJ, Mata M (2006b) Tumor necrosis factor-alpha contributes to below-level neuropathic pain after spinal cord injury. Ann Neurol 59:843-851.

Perez Velazquez JL, Frantseva MV, Carlen PL (1997) In vitro ischemia promotes glutamate-mediated free radical generation and intracellular calcium accumulation in hippocampal pyramidal neurons. J Neurosci 17:9085-9094.

Pickett GE, Campos-Benitez M, Keller JL, Duggal N (2006) Epidemiology of traumatic spinal cord injury in Canada. Spine 31:799-805.

Picklo MJ, Amarnath V, McIntyre JO, Graham DG, Montine TJ (1999) 4-Hydroxy-2(E)-nonenal inhibits CNS mitochondrial respiration at multiple sites. J Neurochem 72:1617-1624.

Pikov V, Gillis RA, Jasmin L, Wrathall JR (1998) Assessment of lower urinary tract functional deficit in rats with contusive spinal cord injury. J Neurotrauma 15:375-386.

Pithon-Curi TC, De Melo MP, Curi R (2004) Glucose and glutamine utilization by rat lymphocytes, monocytes and neutrophils in culture: a comparative study. Cell Biochem Funct 22:321-326.

Pithon-Curi TC, Trezena AG, Tavares-Lima W, Curi R (2002a) Evidence that glutamine is involved in neutrophil function. Cell Biochem Funct 20:81-86.

Pithon-Curi TC, Levada AC, Lopes LR, Doi SQ, Curi R (2002b) Glutamine plays a role in superoxide production and the expression of p47phox, p22phox and gp91phox in rat neutrophils. Clin Sci (Lond) 103:403-408.

Pitts RF, Pilkington LA, MacLeod MB, Leal-Pinto E (1972) Metabolism of glutamine by the intact functioning kidney of the dog. Studies in metabolic acidosis and alkalosis. J Clin Invest 51:557-565.

Plemel JR, Duncan G, Chen KW, Shannon C, Park S, Sparling JS, Tetzlaff W (2008) A graded forceps crush spinal cord injury model in mice. J Neurotrauma 25:350-370.

Popovich PG, Longbrake EE (2008) Can the immune system be harnessed to repair the CNS? Nat Rev Neurosci 9:481-493.

Popovich PG, Wei P, Stokes BT (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 377:443-464.

Popovich PG, Guan Z, Wei P, Huitinga I, van Rooijen N, Stokes BT (1999) Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp Neurol 158:351-365.

Popovich PG, Guan Z, McGaughy V, Fisher L, Hickey WF, Basso DM (2002) The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. J Neuropathol Exp Neurol 61:623-633.

Profyris C, Cheema SS, Zang D, Azari MF, Boyle K, Petratos S (2004) Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol Dis 15:415-436.

Rae C, Hare N, Bubb W, McEwan S, Broer A, McQuillan J, Balcar V, Conigrave A, Broer S (2003) Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. J Neurochem 85:503-514.

Rao SD, Yin HZ, Weiss JH (2003) Disruption of glial glutamate transport by reactive oxygen species produced in motor neurons. J Neurosci 23:2627-2633.

Rapalino O, Lazarov-Spiegler O, Agranov E, Velan GJ, Yoles E, Fraidakis M, Solomon A, Gepstein R, Katz A, Belkin M, Hadani M, Schwartz M (1998) Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat Med 4:814-821.

Rauchova H, Ledvinkova J, Kalous M, Drahota Z (1995) The effect of lipid peroxidation on the activity of various membrane-bound ATPases in rat kidney. Int J Biochem Cell Biol 27:251-255.

Richards DA, Tolias CM, Sgouros S, Bowery NG (2003) Extracellular glutamine to glutamate ratio may predict outcome in the injured brain: a clinical microdialysis study in children. Pharmacol Res 48:101-109.

Rivlin A, Tator C (1978a) Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. Surg Neurol 10:38-43.

Rivlin AS, Tator CH (1977) Objective clinical assessment of motor function after experimental spinal cord injury in the rat. J Neurosurg 47:577-581.

Rivlin AS, Tator CH (1978b) Regional spinal cord blood flow in rats after severe cord trauma. J Neurosurg 49:844-853.

Rogeri P, Rosa LC (2005) Plasma glutamine concentration in spinal cord injured patients. Life Sci 77:2351-2360.

Ronne Engstrom E, Hillered L, Enblad P, Karlsson T (2005) Cerebral interstitial levels of glutamate and glutamine after intravenous administration of nutritional amino acids in neurointensive care patients. Neurosci Lett 384:7-10.

Rose RC, Bode AM (1995) Comments on the glutathione-ascorbic acid redox couple. Free Radic Biol Med 18:955-956.

Roth E, Oehler R, Manhart N, Exner R, Wessner B, Strasser E, Spittler A (2002) Regulative potential of glutamine--relation to glutathione metabolism. Nutrition 18:217-221.

Rothman DL, Sibson NR, Hyder F, Shen J, Behar KL, Shulman RG (1999) In vivo nuclear magnetic resonance spectroscopy studies of the relationship between the glutamate-glutamine neurotransmitter cycle and functional neuroenergetics. Philos Trans R Soc Lond B Biol Sci 354:1165-1177.

Rouse K, Nwokedi E, Woodliff JE, Epstein J, Klimberg VS (1995) Glutamine Enhances Selectivity of Chemotherapy Through Changes in Glutathione Metabolism. Ann Surg 221:420-426.

Ruifrok AC, Johnston DA (2001) Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 23:291-299.

Sandler AN, Tator CH (1976) Effect of acute spinal cord compression injury on regional spinal cord blood flow in primates. J Neurosurg 45:660-676.

Saruhashi Y, Young W (1994) Effect of mianserin on locomotory function after thoracic spinal cord hemisection in rats. Exp Neurol 129:207-216.

Savarese DM, Savy G, Vahdat L, Wischmeyer PE, Corey B (2003) Prevention of chemotherapy and radiation toxicity with glutamine. Cancer Treat Rev 29:501-513.

Sayer FT, Kronvall E, Nilsson OG (2006) Methylprednisolone treatment in acute spinal cord injury: the myth challenged through a structured analysis of published literature. Spine J 6:335-343.

Scheff SW, Saucier DA, Cain ME (2002) A statistical method for analyzing rating scale data: the BBB locomotor score. J Neurotrauma 19:1251-1260.

Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lumpp JE, Jr. (2003) Experimental modeling of spinal cord injury: characterization of a force-defined injury device. J Neurotrauma 20:179-193.

Schloerb PR, Skikne BS (1999) Oral and parenteral glutamine in bone marrow transplantation: a randomized, double-blind study. JPEN J Parenter Enteral Nutr 23:117-122.

Schulman AS, Willcutts KF, Claridge JA, O'Donnell KB, Radigan AE, Evans HL, McElearney ST, Hedrick TL, Lowson SM, Schirmer BD, Young JS, Sawyer RG (2006) Does enteral glutamine supplementation decrease infectious morbidity? Surg Infect (Larchmt) 7:29-35.

Schulman AS, Willcutts KF, Claridge JA, Evans HL, Radigan AE, O'Donnell KB, Camden JR, Chong TW, McElearney ST, Smith RL, Gazoni LM, Farinholt HM, Heuser CC, Lowson SM, Schirmer BD, Young JS, Sawyer RG (2005) Does the addition of glutamine to enteral feeds affect patient mortality? Crit Care Med 33:2501-2506.

Schultke E, Kendall E, Kamencic H, Ghong Z, Griebel RW, Juurlink BH (2003) Quercetin promotes functional recovery following acute spinal cord injury. J Neurotrauma 20:583-591.

Schulz JB, Lindenau J, Seyfried J, Dichgans J (2000) Glutathione, oxidative stress and neurodegeneration. Eur J Biochem 267:4904-4911.

Schwartz M, Yoles E (2006) Immune-based therapy for spinal cord repair: autologous macrophages and beyond. J Neurotrauma 23:360-370.

Schwartz M, Lazarov-Spiegler O, Rapalino O, Agranov I, Velan G, Hadani M (1999) Potential repair of rat spinal cord injuries using stimulated homologous macrophages. Neurosurgery 44:1041-1045; discussion 1045-1046.

Sekhon LH, Fehlings MG (2001) Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine 26:S2-12.

Sharma HS, Sjoquist PO, Mohanty S, Wiklund L (2006) Post-injury treatment with a new antioxidant compound H-290/51 attenuates spinal cord trauma-induced c-fos expression, motor dysfunction, edema formation, and cell injury in the rat. Acta Neurochir Suppl 96:322-328.

Sheng H, Wang H, Homi HM, Spasojevic I, Batinic-Haberle I, Pearlstein RD, Warner DS (2004) A no-laminectomy spinal cord compression injury model in mice. J Neurotrauma 21:595-603.

Shohami E, Gati I, Beit-Yannai E, Trembovler V, Kohen R (1999) Closed head injury in the rat induces whole body oxidative stress: overall reducing antioxidant profile. J Neurotrauma 16:365-376.

Shulman RG, Rothman DL (1998) Interpreting functional imaging studies in terms of neurotransmitter cycling. Proc Natl Acad Sci U S A 95:11993-11998.

Silberstein M, McLean K (1994) Non-contiguous spinal injury: clinical and imaging features, and postulated mechanism. Paraplegia 32:817-823.

Simmons TW, Jamall IS (1988) Significance of alterations in hepatic antioxidant enzymes. Primacy of glutathione peroxidase. Biochem J 251:913-917.

Springer JE, Azbill RD, Mark RJ, Begley JG, Waeg G, Mattson MP (1997a) 4-hydroxynonenal, a lipid peroxidation product, rapidly accumulates following traumatic spinal cord injury and inhibits glutamate uptake. J Neurochem 68:2469-2476.

Springer JE, Azbill RD, Mark RJ, Begley JG, Waeg G, Mattson MP (1997b) 4-hydroxynonenal, a lipid peroxidation product, rapidly accumulates following traumatic spinal cord injury and inhibits glutamate uptake. J Neurochem 68:2469-2476.

Steedman WM, Molony V, Iggo A (1985) Nociceptive neurones in the superficial dorsal horn of cat lumbar spinal cord and their primary afferent inputs. Exp Brain Res 58:171-182.

Stumvoll M, Perriello G, Meyer C, Gerich J (1999) Role of glutamine in human carbohydrate metabolism in kidney and other tissues. Kidney Int 55:778-792.

Stumvoll M, Meyer C, Perriello G, Kreider M, Welle S, Gerich J (1998) Human kidney and liver gluconeogenesis: evidence for organ substrate selectivity. Am J Physiol 274:E817-826.

Subramaniam R, Roediger F, Jordan B, Mattson MP, Keller JN, Waeg G, Butterfield DA (1997) The lipid peroxidation product, 4-hydroxy-2-trans-nonenal, alters the conformation of cortical synaptosomal membrane proteins. J Neurochem 69:1161-1169.

Sullivan PG, Krishnamurthy S, Patel SP, Pandya JD, Rabchevsky AG (2007) Temporal characterization of mitochondrial bioenergetics after spinal cord injury. J Neurotrauma 24:991-999.

Takami T, Oudega M, Bethea JR, Wood PM, Kleitman N, Bunge MB (2002) Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome. J Neurotrauma 19:653-666.

Tal M (1999) A Role for Inflammation in Chronic Pain. Curr Rev Pain 3:440-446.

Tamarappoo BK, Raizada MK, Kilberg MS (1997) Identification of a system N-like Na(+)-dependent glutamine transport activity in rat brain neurons. J Neurochem 68:954-960.

Tan S, Wood M, Maher P (1998) Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem 71:95-105.

Tanhoffer RA, Yamazaki RK, Nunes EA, Pchevozniki AI, Pchevozniki AM, Nogata C, Aikawa J, Bonatto SJ, Brito G, Lissa MD, Fernandes LC (2007) Glutamine concentration and immune response of spinal cord-injured rats. J Spinal Cord Med 30:140-146.

Taoka Y, Ikata T, Fukuzawa K (1990) Influence of dietary vitamin E deficiency on compression injury of rat spinal cord. J Nutr Sci Vitaminol (Tokyo) 36:217-226.

Taoka Y, Okajima K, Uchiba M, Johno M (2001) Methylprednisolone reduces spinal cord injury in rats without affecting tumor necrosis factor-alpha production. J Neurotrauma 18:533-543.

Taoka Y, Okajima K, Uchiba M, Murakami K, Kushimoto S, Johno M, Naruo M, Okabe H, Takatsuki K (1997) Role of neutrophils in spinal cord injury in the rat. Neuroscience 79:1177-1182.

Tator CH (1983) Spine-spinal cord relationships in spinal cord trauma. Clin Neurosurg 30:479-494.

Tator CH (1998) Biology of neurological recovery and functional restoration after spinal cord injury. Neurosurgery 42:696-707; discussion 707-698.

Tator CH (2006) Review of treatment trials in human spinal cord injury: issues, difficulties, and recommendations. Neurosurgery 59:957-982; discussion 982-957.

Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75:15-26.

Tegeder I, Niederberger E, Schmidt R, Kunz S, Guhring H, Ritzeler O, Michaelis M, Geisslinger G (2004) Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. J Neurosci 24:1637-1645.

Tildon JT, Roeder LM (1984) Glutamine oxidation by dissociated cells and homogenates of rat brain: kinetics and inhibitor studies. J Neurochem 42:1069-1076.

Tildon JT, Roeder LM, Stevenson JH (1985) Substrate oxidation by isolated rat brain mitochondria and synaptosomes. J Neurosci Res 14:207-215.

Trivedi A, Olivas AD, Noble-Haeusslein LJ (2006) Inflammation and Spinal Cord Injury: Infiltrating Leukocytes as Determinants of Injury and Repair Processes. Clin Neurosci Res 6:283-292.

Tyurin V, Tyurina Y, Borisenko G, Sokolova T, Ritov V, Quinn P, Rose M, Kochanek P, Graham S, Kagan V (2000) Oxidative stress following traumatic brain injury in rats: quantitation of biomarkers and detection of free radical intermediates. J Neurochem 75:2178-2189.

Vaiva G, Boss V, Ducrocq F, Fontaine M, Devos P, Brunet A, Laffargue P, Goudemand M, Thomas P (2006) Relationship between posttrauma GABA plasma levels and PTSD at 1-year follow-up. Am J Psychiatry 163:1446-1448.

Vale FL, Burns J, Jackson AB, Hadley MN (1997) Combined medical and surgical treatment after acute spinal cord injury: results of a prospective pilot study to assess the merits of aggressive medical resuscitation and blood pressure management. J Neurosurg 87:239-246.

Van Der Hulst RR, Deutz NE, Von Meyenfeldt MF, Elbers JM, Stockbrugger RW, Soeters PB (1994) Decrease of mucosal glutamine concentration in the nutritionally depleted patient. Clin Nutr 13:228-233.

Vogel LC, Klaas SJ, Lubicky JP, Anderson CJ (1998) Long-term outcomes and life satisfaction of adults who had pediatric spinal cord injuries. Arch Phys Med Rehabil 79:1496-1503.

Waagepetersen HS, Sonnewald U, Schousboe A (2003) Compartmentation of glutamine, glutamate, and GABA metabolism in neurons and astrocytes: functional implications. Neuroscientist 9:398-403.

Wallace MC, Tator CH, Frazee P (1986) Relationship between posttraumatic ischemia and hemorrhage in the injured rat spinal cord as shown by colloidal carbon angiography. Neurosurgery 18:433-439.

Wang S, Wang G, Barton BE, Murphy TF, Huang HF (2006) Beneficial Effects of Vitamin E in Sperm Functions in the Rat after Spinal Cord Injury. J Androl.

Watford M (1994) Glutamine metabolism in rat small intestine: synthesis of three-carbon products in isolated enterocytes. Biochim Biophys Acta 1200:73-78.

Watkins LR, Milligan ED, Maier SF (2003) Glial proinflammatory cytokines mediate exaggerated pain states: implications for clinical pain. Adv Exp Med Biol 521:1-21.

Weaver LC, Gris D, Saville LR, Oatway MA, Chen Y, Marsh DR, Hamilton EF, Dekaban GA (2005) Methylprednisolone causes minimal improvement after spinal cord injury in rats, contrasting with benefits of an anti-integrin treatment. J Neurotrauma 22:1375-1387.

Welbourne TC (1987) Interorgan glutamine flow in metabolic acidosis. Am J Physiol 253:F1069-1076.

Welbourne TC (1988) Role of the lung in glutamine homeostasis. Contrib Nephrol 63:178-182.

Williamson JM, Boettcher B, Meister A (1982) Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proc Natl Acad Sci U S A 79:6246-6249.

Wilmore DW (2001) The effect of glutamine supplementation in patients following elective surgery and accidental injury. J Nutr 131:2543S-2549S; discussion 2550S-2541S.

Wischmeyer PE (2006) The glutamine story: where are we now? Curr Opin Crit Care 12:142-148.

Wischmeyer PE (2008) Glutamine: role in critical illness and ongoing clinical trials. Curr Opin Gastroenterol 24:190-197.

Xu JA, Hsu CY, Liu TH, Hogan EL, Perot PL, Jr., Tai HH (1990) Leukotriene B4 release and polymorphonuclear cell infiltration in spinal cord injury. J Neurochem 55:907-912.

Xu W, Chi L, Xu R, Ke Y, Luo C, Cai J, Qiu M, Gozal D, Liu R (2005) Increased production of reactive oxygen species contributes to motor neuron death in a compression mouse model of spinal cord injury. Spinal Cord 43:204-213.

Yang R, Martin-Hawver L, Woodall C, Thomas A, Qureshi N, Morrison D, Van Way C, 3rd (2007) Administration of glutamine after hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. JPEN J Parenter Enteral Nutr 31:94-100.

Yoshida S, Kaibara A, Ishibashi N, Shirouzu K (2001) Glutamine supplementation in cancer patients. Nutrition 17:766-768.

Young VR, Ajami AM (2001) Glutamine: the emperor or his clothes? J Nutr 131:2449S-2459S; discussion 2486S-2447S.

Young W, Yen V, Blight A (1982) Extracellular calcium ionic activity in experimental spinal cord contusion. Brain Res 253:105-113.

Zalkin H, Smith JL (1998) Enzymes utilizing glutamine as an amide donor. Adv Enzymol Relat Areas Mol Biol 72:87-144.

Ziv Y, Yaniv, Avidan H, Hila , Pluchino S, Stefano, Martino G, Gianvito, Schwartz M, Michal (2006) Synergy between immune cells and adult neural stem/progenitor cells promotes functional recovery from spinal cord injury. Proceedings of the National Academy of Sciences of the United States of America 103:13174-13179.

APPENDIX A

ADDRESSING STATISTICAL ISSUES AND DEMONSTRATING ADDITIONAL STRATEGIES FOR BEHAVIOURAL DATA ANALYSIS

The BBB scoring system is an ordinal scale in which the improvements in function are not linearly distributed within the 22 points of the scale (Basso et al., 1995). The initial papers describing the method used the parametric test ANOVA to determine significance, that choice has come into question as a non-parametric test is normally considered the appropriate choice for data from an ordinal scale (Daniel, 1987). The argument has been made that as the BBB scores do not rank the animals but rather assign a score from the scale according to their locomotor ability, that the use of the ANOVA is valid (Scheff et al., 2002). I have found that most articles using the BBB scale have agreed with Scheff et al. and tested for significance with an ANOVA with a post hoc Tukey's test, as I have done in this project. I have however decided to re-examine the data from our largest experiment (Chapter 3, experiment #1) using the non-parametric tests Kruskal-Wallis and Mann Whitney to determine if differences between methods would have changed our interpretations.

The Kruskal-Wallis test found a significant difference within the BBB data comparing saline and glutamine treated animals with a P value of 0.001. A Mann-Whitney test was then used on each injury severity to compare data from saline and glutamine treated animals. The results comparing the findings using ANOVA and the Mann-Whitney were identical. The same statistical differences between the saline and glutamine treated samples for the 0.4, 0.6 and 0.8 mm injuries were found, in addition to the non-significant changes in the 0.2 and 1.0 mm injury groups.

In addition, a suggestion was made to re-analyze the BBB scores by establishing particular landmarks in locomotor recovery corresponding to BBB scores and count the number of weeks each animal reaches or exceeds this score. The two landmarks chosen for this data analysis were the "extensive movement of all three joints of the hind limb"

(BBB = 7) and “plantar placement of the paw with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping” which coincides with a BBB score of 9 (Basso et al., 1995). The only statistically significant difference found was between the saline and glutamine treated animals that received the 0.4 mm compression injury in data examining weeks at a BBB score of 7 or higher (Figure A1).

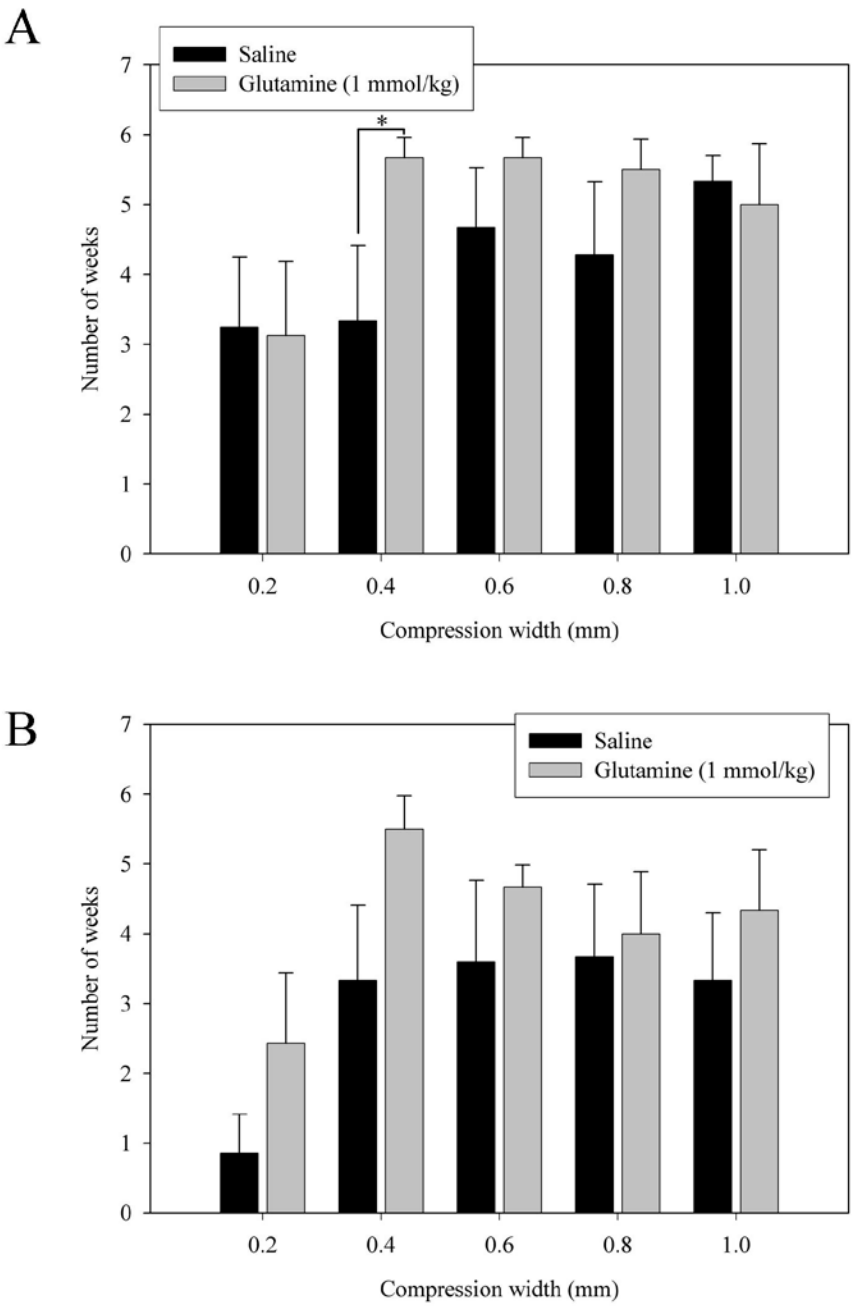


Figure A1: Counting the number of weeks each animal achieves a BBB score of 7 (A) or 9 (B). For each of the two chosen landmarks for recovery, namely BBB scores of 7 or 9, the number of weeks each animal spent at or above that score were counted. Data are expressed as mean ± standard deviation. * indicates a significant difference ($P < 0.05$).

APPENDIX B

SCATTER PLOTS DEMONSTRATION CORRELATIONS FROM CHAPTER 3

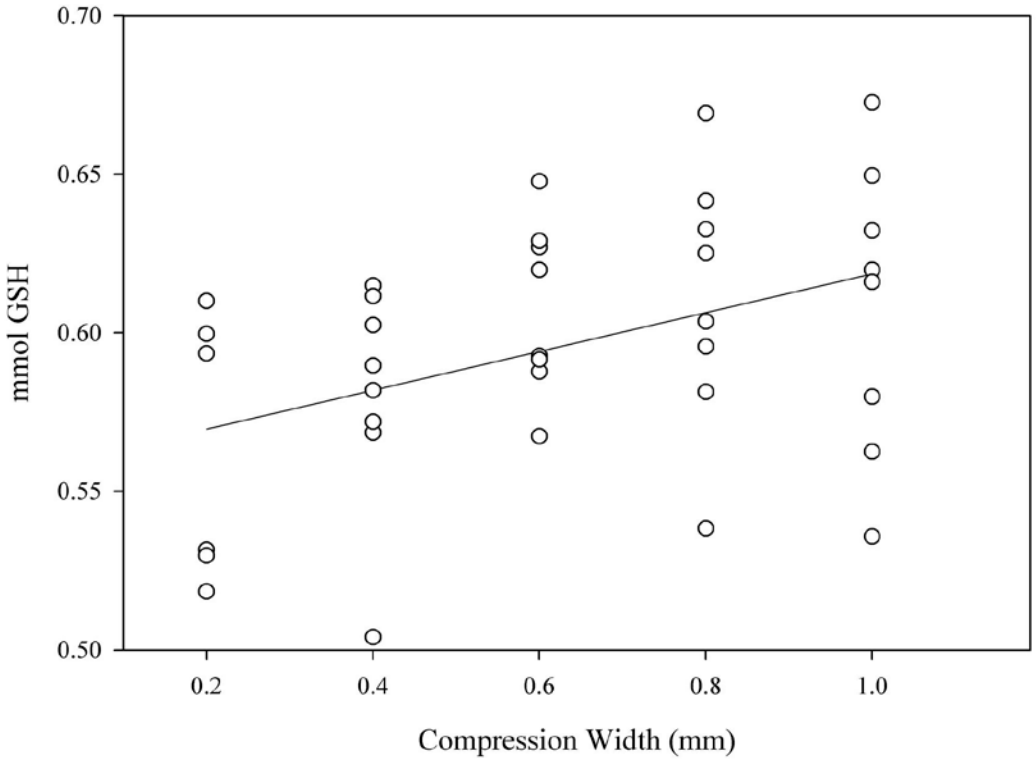


Figure B-1: Scatter plot demonstrating the correlation between injury severity and blood GSH concentrations in samples taken 2 hours after injury. $R^2 = 0.45$. Data represented are raw data.

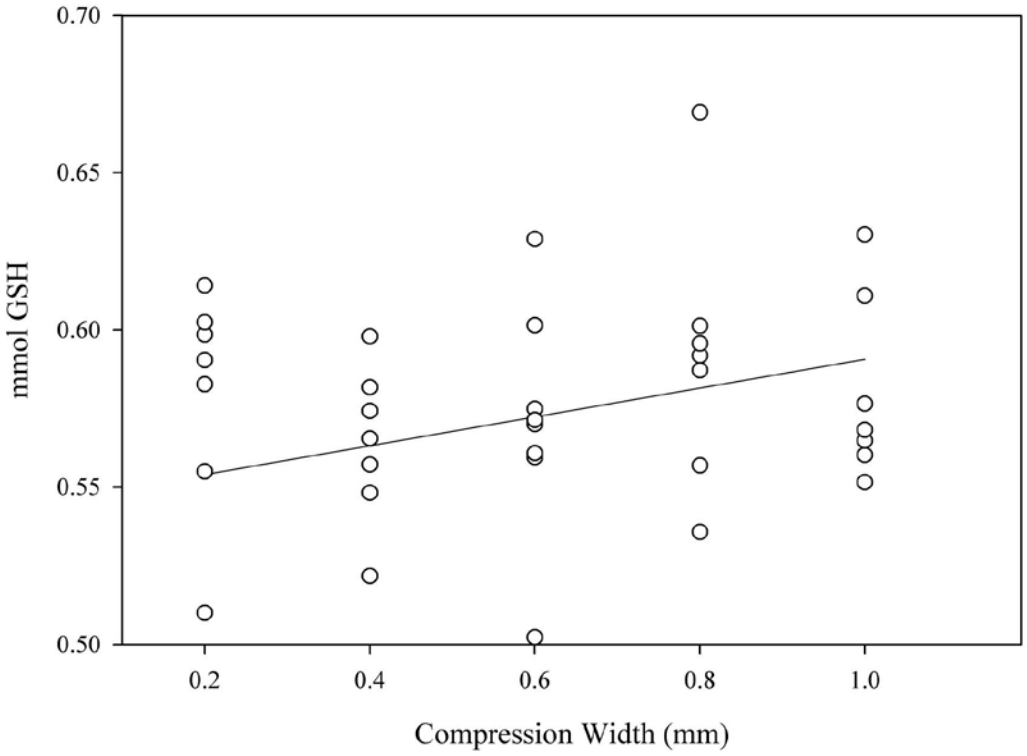


Figure B-2: Scatter plot demonstrating the correlation between injury severity and blood GSH concentrations in samples taken 25 hours after injury. $R^2 = 0.33$. Data represented are raw data.

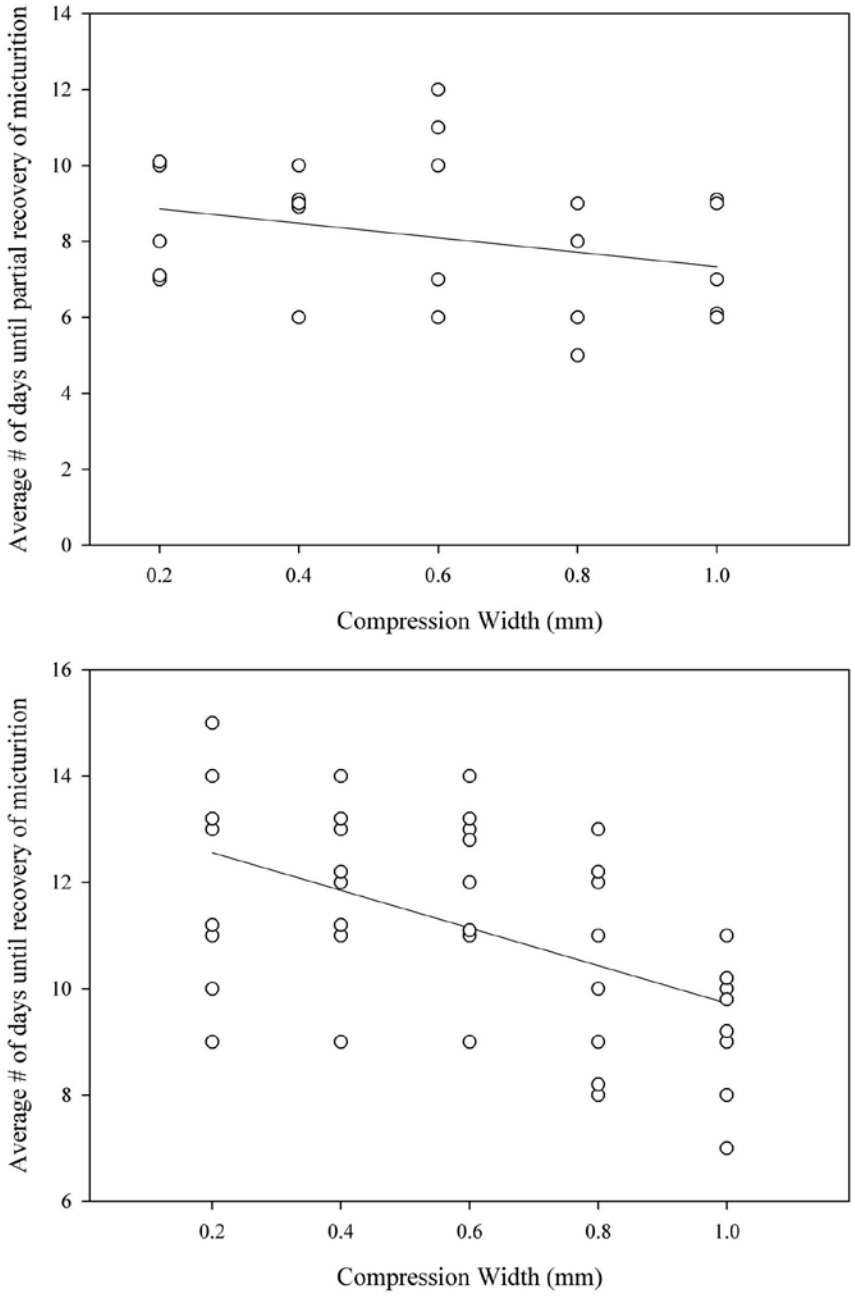


Figure B-3: Scatter plots demonstrating the correlation between injury severity and the number of days before partial recovery (A) or recovery (B) of micturition occurs. A) $R^2 = 0.566$, B) $R^2 = 0.585$. Data represented are raw data.

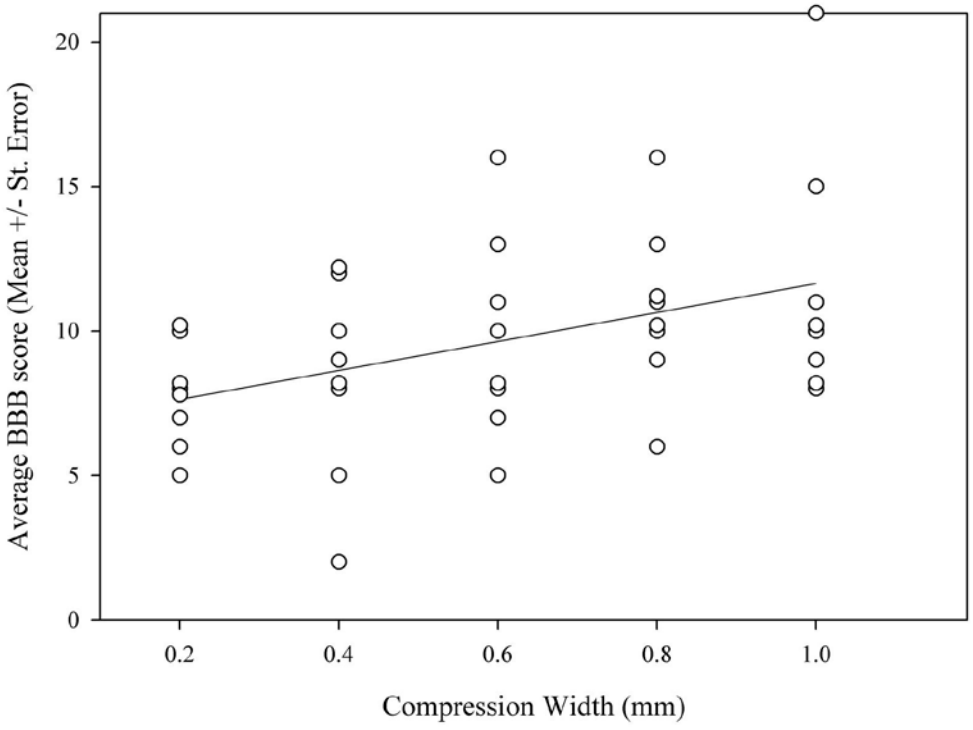


Figure B-4: Scatter plot demonstrating the correlation between injury severity and average BBB score 6 weeks post injury. $R^2 = 0.663$. Data represented are raw data.

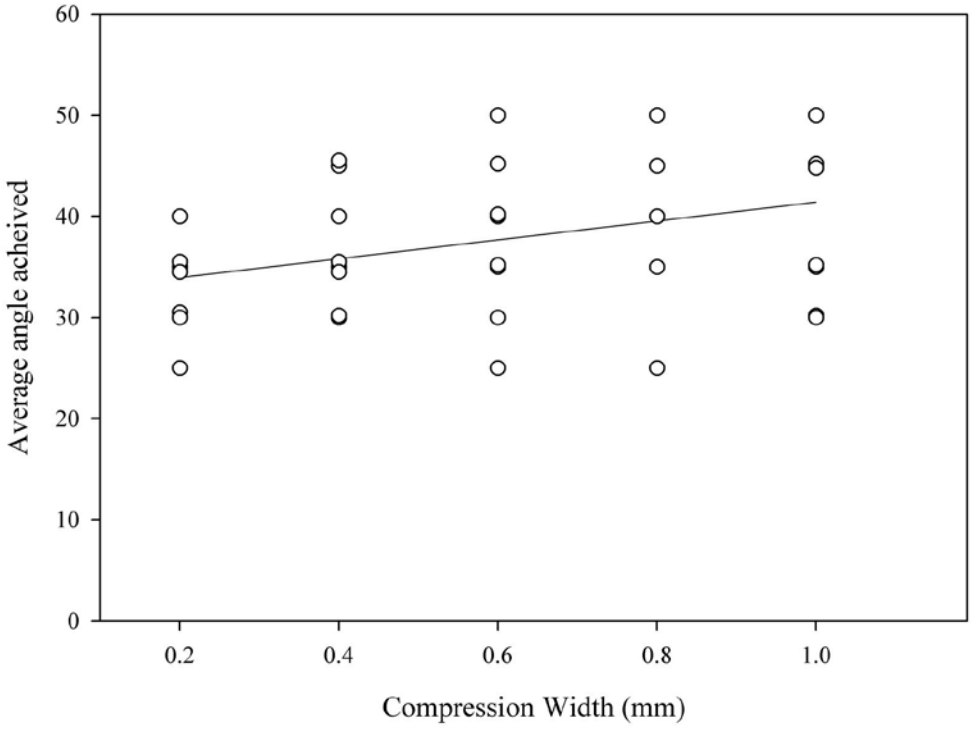


Figure B-5: Scatter plot demonstrating the correlation between injury severity and average angle achieved in the inclined plane test 6 weeks post injury. $R^2 = 0.652$. Data represented are raw data.

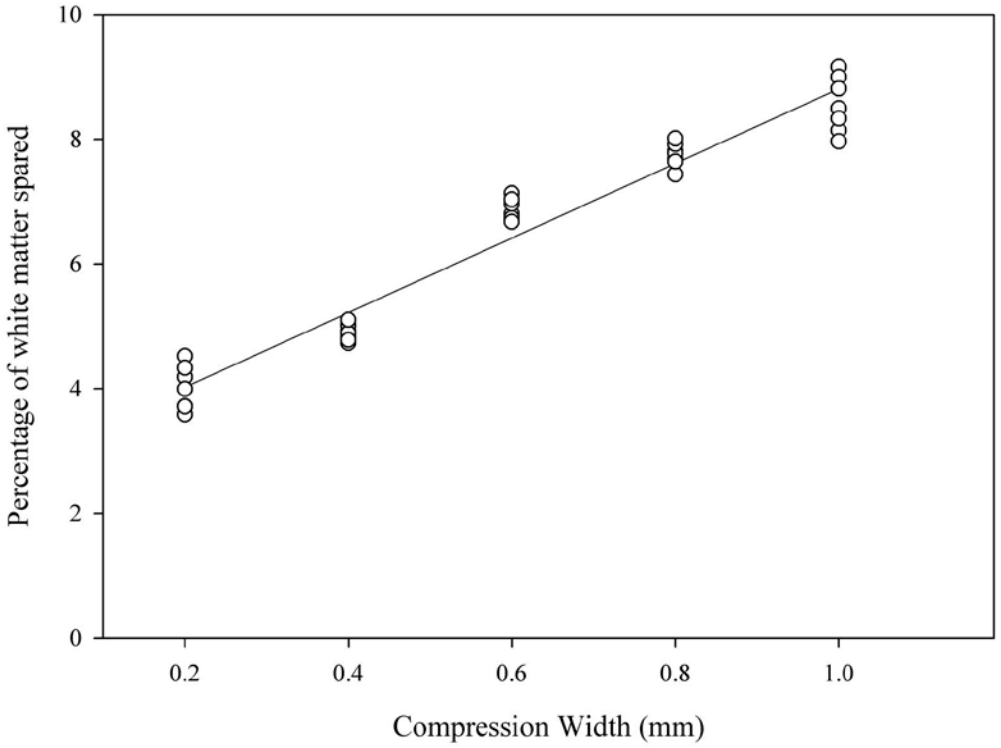


Figure B-6: Scatter plot demonstrating the correlation between injury severity and the percentage of white matter sparing at the epicentre of injury 6 weeks post injury. R^2 = 0.950. Data represented are raw data.