

EFFECTS OF ACUTE LOCOMOTOR TRAINING WITH OR WITHOUT BACLOFEN
THERAPY ON SPASTICITY AFTER CONTUSION SPINAL CORD INJURY (SCI)

By

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by

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To the memory of my mother and grandfather-in-law whom I will miss throughout my life.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis Of Variance
BBB	Basso, Beattie and Bresnahan scale
BDNF	Brain Derived Neurotropic Factor
Ca ²⁺ channels	Calcium channels
Cl ⁻ channels	Chloride channels
CPG	Central Pattern Generator
Deg/sec	Degrees/second
EMG	Electromyeloencephalogram
EPSP's	Excitatory Postsynaptic Potentials
GABA _b	Gamma Amino Butyric Acid
GAP ₄₃	Growth Assosiated Protein 43
IgG	Immunoglobulin G
IHC	Imunohistochemistry
ITB	Intrathecal Baclofen
Kdynes	Kilodynes
MASCIS	Multicenter Animal Spinal Cord Injury Studies
Na ⁺ channels	Sodium channels
PAD	Primary Afferent Depolarization
PIC	Persistent Inward Currents
SCI	Spinal Cord Injury
VDAT	Velocity Dependent Ankle torque
5-HT	5-Hydroxytryptamine (Serotonin)

Abstract of Thesis Presented to the Graduate School
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Progress has been made in the therapeutic treatment of spasticity, especially utilizing intrathecal baclofen (ITB). In addition to medical treatment, two decades of studies in animals and humans report exciting possibilities for locomotor training to improve locomotor recovery. However, many unknowns remain regarding the timing of treatments, and whether they interact to facilitate or inhibit rehabilitation directed at recovery of voluntary motor activity. Since spasticity development is progressive, we explored the potential for acute initiation of therapies to influence the development of spasticity. These studies were performed to evaluate the safety, feasibility, and efficacy of two early intervention treatments (performed alone, or in combination) on measures of spasticity and long term functional outcome measures following midthoracic contusion SCI. Four groups of animals received contusion injuries to the midthoracic spinal cord using the New York University (NYU) impounder and the Multicenter Animal Spinal Cord Injury Studies (MASCIS) protocol for moderate injury (10 gm weight drop at a distance of 12.5mm). Two groups of animals received ITB pumps (Azla Corp., Palo Alto, CA) at the time of injury; the other two groups received pumps with saline vehicle. The tip of the intrathecal cannula was placed in the subdural space of the L₁-L₂ lumbar spinal cord. Beginning

at day 1 following injury, two 20 minutes sessions of locomotor exercise were performed using a custom made cycle locomotor trainer on one ITB and one vehicle control group. Velocity dependent ankle torque, ankle extensor muscle EMGs, hindlimb axis, and open field locomotion (BBB), were recorded in all groups. The data indicate that at 2 months following injury, cycle training alone produced a greater reduction in ankle torque than baclofen alone. However, ankle extensor spasticity was significantly lower, and rate of open field recovery was greatest in the animals that received the combination of ITB and cycle training, compared with animals that receiving no treatment or either of the treatments performed alone. No between group differences were observed in footprint data for acute treatments alone or combined. Terminal Immunohistochemistry of lumbar spinal cord segments revealed increased expression of markers for GABA (GABA_b, GAD₆₇) & monoamines (DBH (NE), and 5-HT) in combined treated animals. Treatment related upregulation of GABA/GABA_b molecules might exert significant roles in pre-synaptic inhibition and upregulated monomamines might prevent supersensitivity of monoamine receptors (α-1 & 5-HT) and thus might block both the early onset and the late onset of spasticity. These data indicate that acute treatments using locomotor exercise / ITB are safe and feasible initiated as early as postcontusion day 1 and ITB and locomotor treatments differentially influenced early and late onset of the development of spasticity.

CHAPTER 1 INTRODUCTION

Spinal Cord Injury

The spinal cord (SC) is a vital organ of the body, which is necessary for the maintenance of posture and locomotion. Trauma to the SC leads to a series of events resulting in changes in reflex excitability from spinal shock to a hyperreflexive condition known as spasticity. Frequent causes of SC damage are trauma (car accident, gunshot, falls, etc.) or disease (polio, spina bifida, Friedreich's Ataxia, etc.). The SC does not have to be severed in order for a loss of functioning to occur. In fact, in most people with spinal cord injury (SCI), the SC is intact, but the damage to it results in loss of functioning. About half of the patients with SC cord trauma have incomplete injuries without any signs of voluntary motor or sensory perception below the level of the lesion. The victims of cord trauma are mainly young men in their early 20s to 30s (National Spinal Cord Injury Statistical Center). Efforts should be made to improve the quality of life of the young victims and to reduce the clinical burden in society. To date, no suitable therapy for the victims of SCI is available. New therapeutic strategies with the possibility of regeneration of the lesioned SC axons are needed to improve the quality of life of patients with SCI. Spinal cord injury induces secondary biochemical responses that include both neurotoxic and neuroprotective processes (Tator,1995). It is believed that the balance between these reactions in part determines ultimate tissue damage and the degree of associated neurological recovery. Although functional regeneration appears to be limited after SCI in mammals, it is not unlikely that plasticity of surviving cells contributes to functional recovery that is often observed.

Spasticity

Spasticity is a form of muscular hypertonia, due to a velocity-dependent increase in tonic stretch reflexes during passive movement, which results from abnormal spinal processing of

proprioception after SCI. Spasticity, is a major health problem for patients with a SCI. It limits patients' mobility and affects their independence in activities of daily living and work. Spasticity may also cause pain, loss of range of motion, contractures, sleep disorders and impair ambulation in patients with an incomplete lesion. The effectiveness of available drugs is still uncertain and they may cause adverse effects. Assessing what works in this area is complicated by the lack of valid and reliable measurement tools (Taricco et al. 2006).

The present treatment for spasticity is the use of anti-spastic drugs such as baclofen, which is a GABA_b agonist. But the appropriate dose and the time frame at which it should be started to treat spasticity is not known. In humans, baclofen is given chronically i.e., 1 month after the injury, as the FDA does not approve of its use acutely. Whether baclofen given acutely after SCI can prevent the development of spasticity is not known. It is not known that acute administration of baclofen administration is safe. In animals and humans following SCI, spasticity appears at a chronic stage. One of the primary goals of my thesis was to obtain relevant information regarding baclofen's safety and efficacy in the acute stage, before the onset of spasticity. Rehabilitation in the form of treadmill training is widely used in human SCI cases to improve their gait. Treadmill training in humans is laborious and requires a lot of manpower. Setups are available in only a few centers across the nation, so the training is not accessible to all the patients. The purpose of this study was also to test the hypothesis that a customized motorized bicycle could be used instead of the treadmill to treat spasticity in the acute stage and to maintain the feasibility and safety of the patient without any deterioration in his condition. This study compared combination therapy of drug (baclofen) and locomotor training (bicycle) with individual therapy, started acutely after midthoracic contusion SCI in animal model, and to test if

combination therapy, started acutely, is more useful in preventing the development of spasticity and in improving gait.

The wide range of outcome measures of spasticity and gait and comparison of two treatment modalities (locomotor training and drug therapy) proposed in this study might provide translational data which ultimately influence the quality of SCI patients. These studies propose a strategy to utilize quantitative measures of behavior, neurophysiology, IHC, and imaging (MRI) techniques to further increase our understanding of neurobiology of spasticity and gait following SCI and locomotor rehabilitation. Translation of these findings may provide safe, timely, and effective intervention strategies and evidence-based resources for translatable therapeutic design, which can ultimately benefit the SCI patient.

CHAPTER 2 BACKGROUND

This chapter presents the relevant background of SCI induced spasticity and gait problems as well as present treatment modalities to deal with these clinical problems. The scientific society studying SCI uses various types of injury models in animals to mimic the type of injury seen in humans but the most commonly used method for SCI is mid-thoracic contusion SCI which has documented to develop spasticity, even though there are various other types of injury models which are presented below:

Recent studies of SCI models have indicated an increasing need for a more thorough understanding of the multiple components of spinal cord segmental plasticity for which therapeutic interventions can be rationally targeted by various interventions, including locomotor training (Raineteau, & Schwab 2001). In that context, this thesis work was focused on spasticity—a common aftermath of SCI – that reflects maladaptive changes in spinal cord circuitry affecting motoneuron excitability and output. Spasticity is often one of the most difficult neurological consequences to manage. Therefore, studies that can lead to a more in-depth appreciation of the underpinnings of spasticity and therapies that can attenuate its impact are of great importance. Accordingly, this project addresses locomotor training rehabilitation with or without drug (baclofen) therapy to test the effect(s) on spasticity using multidisciplinary analytical approaches.

Spinal Cord Injury Animal Models

Animal models of SCI can be used to study the lesion development, study the mechanism of recovery, and to develop therapeutic intervention. The experimental SCI animal models include the transection, isolation and contusion.

Spinal Cord Contusion Model (Incomplete Spinal Cord Injury)

Basically, human spinal cord injuries are caused by transient compression or contusion of the spinal cord even though penetrating wounds of the spinal cord can result from a knife or gunshot. So, we used animal spinal cord contusion models to study the pathophysiology of SCI and its rehabilitation as it mimics natural human SCI. Most therapies that have gone to human trial were first validated in spinal cord contusion models. Weight drop contusion model was used by Wrathall (1985) and described morphological and behavioral changes in that model; she also developed a combined behavioral score to assess motor, sensory and locomotor changes. Patients with complete spinal cord lesions never improve up to the stage of being able to walk without the assistance of the weight support system and consequently are not able to step on a static floor (Van de Crommert et al. 1998). The inability of patients with complete transections to achieve unassisted walking, unlike the fully spinalized cat, suggests that the greater improvement observed in subjects with incomplete lesions may not solely be attributable to spinal mechanisms, since generation of stepping is probably more dependent on supraspinal and/or proprioceptive inputs in humans than in cat (Edgerton et al. 2001; Van de Crommert et al. 1998). Considering these clinical and functional data and knowing that the ratio of incomplete versus complete spinal cord lesions is becoming increasingly known in the population of paraplegics (Tator et al. 1993), experimental studies using animals with incomplete spinal cord injuries appear clinically and pathophysiologically relevant (Multon et al. 2003).

Spinal Transection Model (Complete Spinal Cord Injury)

In this model there is complete damage to both the descending and ascending fibers and there is no connection between the caudal part of the spinal cord and the brain and disrupts all the neurophil at the injury site. This type of injury is less common in humans as the human spinal cord is surrounded by vertebrae, tissues and muscles that provide protection to the spinal cord.

Spinal Isolation Model

In this model the lumbar region of the spinal cord is functionally isolated via complete spinal cord transection at two sites; this model eliminates supraspinal, infraspinal and peripheral afferent input to motoneurons located in the isolated cord segments while leaving the motoneuron skeletal muscle fiber connections intact. However this model is not used in experimental set up, as cases of human isolation models are not seen frequently so the observation in animal experiments cannot be implemented in human subjects as it can be done in contusion animal models experiment.

Anatomical (Neuromodulatory) Changes Following SCI

Midthoracic spinal cord contusion injury is known to disrupt connectivity to and from the lumbar spinal cord which hosts important segmental circuitry that:

- 1) initiates and sequences lower limb locomotor behavior (central pattern generator, CPG)
- 2) governs reflex excitability (Wrathall et al. 1985, Thompson et al. 1992; 1998).

Impact injury of the thoracic spinal cord produces early increase followed by significant decrease in the monoamines (dopamine, norepinephrine and serotonin), measured below the site of injury. (Thompson et al. 1999). These neuromodulators play an important role individually and interactively in regulation of sensory transmission and the excitability of interneurons, fusimotor and alpha motoneurons (Gladden et al. 1998; 2000). It is known that locomotor exercise increases the expression of norepinephrine in the central nervous system (Dishman et al. 1997). The unifying strategy of exercise based locomotor therapy relates to the hypothesis that repetitive activity induced by training initiates neuronal activity in proper sequence that optimizes the utilization of the diminished but residual central nervous system.

A Rodent Model of Post-SCI Spasticity

The availability of a reliable animal model of post-SCI spasticity is prerequisite to this research. Ideally, the model should be amenable to applications of rigorous outcome measures and test approaches that can have direct translational potential. Previous use of a rodent midthoracic contusion injury paradigm has revealed significant neurophysiological, locomotor, neuromuscular, and histological changes which have collectively demonstrated the feasibility of reproducing significant features of human spasticity (Bose et al. 2002; Thompson et al. 1992, 1993, and 1998). Thompson-Bose lab developed a novel velocity-dependent ankle torque assessment protocol to reveal bonafide features of spasticity in the rat (Bose et al. 2002; Thompson et al. 1996, 2001a, 2002) and showed that changes in spasticity were time-locked to neurophysiological events in relevant areas of the spinal cord. Further, a reflex test protocol (i.e., rate-depression) showed that a fundamental inhibitory process which controls sensory input to hindlimb muscle stretch reflex pathways was significantly decreased following midthoracic contusion injury (Thompson et al. 1992, 1993, 1998). These changes are progressive in onset, severe in magnitude, permanent in duration, and are highly relevant to features observed clinically in humans. Therefore, the methods and model developed in our lab and its employment in this project collectively provide an important and clinically relevant opportunity to investigate issues related to the neurobiology of spasticity and how experimental therapies may modulate this hallmark feature of SCI in a very acute setting.

Mechanism of Spasticity

The onset of spasticity has been correlated in time and intensity to the progressive development of several lasting changes in the excitability of monosynaptic reflexes that compose the neural pathways from the affected muscle stretch receptors to those muscle motor neurons,

(Nielsen et al. 1993). Two premotoneuronal mechanisms have been postulated to account for these changes:

1. multiplication of synaptic input by collateral sprouting of primary afferents (Krenz and Weaver 1998).
2. decreased presynaptic inhibition (Calancie et al. 1993).

Frank and Fuortes (1957) reported that stimulation of group I afferents from flexor muscles was able to depress the Ia monosynaptic EPSPs, without changing the membrane potential, the motoneuron input resistance, or the generation of antidromic action potentials. The depression of the Ia EPSPs was due to presynaptic inhibition. Eccles and Krnjevic (1959) found that stimulation of specific sensory nerves produced a long lasting depolarization of group I muscle and cutaneous afferents and suggested that this primary afferent depolarization (PAD) was the mechanism for presynaptic inhibition. Rudomin and colleagues localized a circuit of interneurons that received afferent collaterals from primary afferent fibers and convergent inputs from the terminals from descending fibers (Rudomin 1999). They showed the circuit for presynaptic inhibition involved a minimum of two interneurons, the second or last order being GABAergic that made axo-axonic synapses on GABA_a receptors on primary afferent terminals. The activation of these receptors induces an outward Cl⁻ current that produces long lasting PAD. During the time course of the PAD (20-300msecs), the GABA_a mediated conductance changes along the primary afferent membrane decreases the effectiveness of afferent volleys to depolarize the membrane and activate the voltage gated calcium channels that are essential for calcium triggered release of neurotransmitters from the primary afferent terminals. The decrease in transmitter release thereby decreased the amplitude of the excitatory postsynaptic potentials produced during the PAD. The knowledge that descending systems modulated PAD, combined with the demonstration that vibratory inhibition was reduced in spastic patients emphasized the

hypothesis that a major source of spasticity was due to the reduction of GABA_a mediated presynaptic inhibition following interruption of descending modulation of the segmental circuitry that produced PAD. Neurotransmitters like GABA_b, GAD₆₇ also play an important role in the development of spasticity which acts as inhibitory molecules preventing the development of spasticity.

GABA

GABA is a spinal inhibitory interneuronal neurotransmitter. There is a high density of GABAergic immunoreactive cell bodies and terminals predominantly within Lamina I through III of the dorsal horn. Occasionally GABA cells were observed in layers IV, V, VI and X and in the ventral horn. GABA functions in pre and postsynaptic inhibition in the spinal cord, via axoaxonal and axosomatic or axodendritic synapses (Shapiro 1997). In vertebrates 2 major types of GABA receptors (GABA_a and GABA_b) are found. GABA_a contains a transmembrane ion channel that conducts chloride ions and is gated by the binding of two agonist molecules. Activation of GABA_a opens the chloride channel, and the influx of the chloride ions inhibits the neuron by causing hyperpolarization. GABA_b is present at lower levels in the central nervous system than is GABA_a and is coupled to Ca²⁺ or K⁺ channels via second messenger systems. GABA_b can be distinguished from GABA_a by its affinity for the agonist baclofen and its lack of affinity for muscimol and bicuculline. Activation of GABA_b by baclofen decreases Ca⁺ conductance and transmitter release and thus acts an inhibitory molecule in synaptic transmission (Bowery et al. 1980, 1989; Shapiro 1997) and prevents the development of spasticity.

Since GABA is localized to interneurons, reactive synaptogenesis of interneuronal GABAergic neurons can be seen in the spinal cord of spastic animals. Thus this molecule is very important for the proposed research since we use baclofen as an antispastic drug and examine its action on the presynaptic GABA_b receptors.

Present Antispasticity Treatment for Spinal Cord Injury

Baclofen has been used widely as an anti-spasticity drug. Baclofen reduces muscle tone and spasms with similar efficacy in patients with spasticity caused by complete or incomplete SCI, or from cerebral origin (Davidoff 1985; Meythaler et al. 2001). A recent finding has proven that the effect of baclofen is more potent when applied intrathecally than orally (Azouvi et al. 1996). GABA_b receptors are distributed extensively in the spinal cord, especially presynaptically on the primary sensory afferent terminals (Yang et al. 2001). Baclofen, as a potent GABA_b receptor agonist, has been shown to decrease synaptic transmission by binding to the presynaptic GABA_b receptors at the afferent terminal through a second-messenger pathway, ultimately decreasing the calcium influx and neurotransmitter release (Batueva et al. 1999; Miller 1998). In addition, baclofen's binding to the presynaptic GABA_b receptors can also decrease the neurotransmitter release by activating potassium channels (Gage 1992) causing hyperpolarization of the post synaptic membrane, thus contributing to its presynaptic inhibitory effect (Li et al. 2004). In the paper by Li (2004) the author showed that in motor neurons of normal animals (or humans) with intact spinal cord and brain stem, there are voltage-dependent persistent inward currents (PICs) that, once activated, can remain active for many seconds after stimulation, producing sustained depolarizations (plateau potentials) and firing (self-sustained firing), thus greatly increasing their excitability (Gorassini et al. 2002; Lee and Heckman 1998a, b). A PIC is a depolarizing current generated by voltage-sensitive channels; the voltage sensitive channels stay open as long as the membrane potential remains above threshold for their activation (Heckman et al. 2004). The PICs are composed of a low-threshold persistent calcium current, carried by Cav1.3 L-type calcium channels, and a TTX-sensitive persistent sodium current (Lee and Heckman 2001; Li and Bennett 2003). Large PICs are not present in motor neurons immediately after spinal cord injury because of the massive loss of brain-stem-derived

monoamines that normally facilitate PICs (Hounsgaard et al. 1988). Exogenous application of metabotropic receptor agonists (such as 5-HT) can enhance PICs and thus recover plateaus and self-sustained firing after acute spinal transection or in vitro slice injury (Lee and Heckman 1998a, b). Baclofen has been shown recently to decrease the amplitude of these enhanced PICs in motor neurons of turtle spinal cord slices and to decrease spontaneously occurring PICs in deep dorsal horn neurons of turtles and rats (Russo et al. 1998). Thus, raising the possibility that baclofen's clinical action may be partly postsynaptic by decreasing the PICs that play an important role in the production of spasticity (Bennett et al. 2001a, b; Li et al. 2004a). It has been found that GABA_b decreased PIC only in acute SCI not in chronic SCI, it increased the PIC, as chronic spinal rats have a moderately lower (30%) sensitivity to baclofen than do acute spinal rats, which might be due to down regulation/desensitization of GABA_b receptors or decreased background GABA levels after chronic SCI (Li et al. 2004). This finding may justify our hypothesis that baclofen started acutely after SCI can prevent the development of spasticity by reducing the monosynaptic reflex mainly by decreasing presynaptic neurotransmitter release and decreasing the PICs by reducing the Ca²⁺ PICs due to the up- regulation of the GABA_b receptors. Systemic baclofen depressed the monosynaptic excitation of Clarke's column neurons by impulse in muscle and cutaneous afferent fibers (Shapiro 1997).

Baclofen, in addition to GABA_b, can bind to a novel bicuculline-insensitive GABA receptor site on primary afferents of the spinal cord and reduce the amount of transmitter released (Bowery et al. 1980,1984). Baclofen is given in an intrathecal pump (ITB). Orally it cannot easily cross the blood brain barrier and only 1% of the total dose reach the central nervous system and so oral doses are generally twice or thrice the required amount which is not feasible. ITB supplies the exact amount of drug to the appropriate area for the required amount of time, thus enabling the effects of baclofen to be studied after immediate administration and to

see the development of tolerance after withdrawal. The major drawback of baclofen therapy in patients today is that it is started much later after the injury as the Food and Drug Administration does not approve baclofen for acute treatment. Spasticity is usually absent in acutely injured animal preparations, and it only gradually develops with chronic injury (>1 month) (Bennett et al. 1999). Also it has been shown that Baclofen started even one week after injury helps in improving spasticity (Wang et al. 2002). So the basic aim of this project is to acutely start baclofen therapy along with bicycle training or alone to prevent the development of spasticity and to see whether there is rebound spasticity after the baclofen treatment is stopped after a certain period of time, in our case after 1 month.

Locomotor Rehabilitation

Rehabilitation in the form of treadmill or customised bicycle is widely used in human SCI and in animal models. Customized bicycle developed as an alternative modality to treadmill, to train SCI injured rats was used in our project to prove it is as effective as treadmill. But exactly what time period after injury it should be started to prevent the development of spasticity and improve the gait and whether training after injury may have other side effects on the patient's body are not known. So we want to test the hypothesis that bicycle training acutely after injury along with baclofen therapy prevents the development of spasticity. Exercise improves SCI extensively due to its adeptness at enhancing sensory function which is mediated by molecular systems dependent on neurotrophic actions. Voluntary wheel training and forced treadmill exercise increased the expression of BDNF and other neurotropic molecules important in synaptic function and neurite outgrowth in the spinal cord and innervated skeletal muscles (Gomez-Pinilla et al. 2005). There is increasing evidence that the human spinal cord is capable of a significant amount of plasticity and that this plasticity is, to a large extent, driven by activity-dependent processes (Edgerton et al. 1997, 2001). This plasticity may occur at any of

many spinal cord regions or cell types such as motoneurons, premotor pattern-generating neurons, and/or nonneuronal cell types. Studies have shown that locomotor training improves the ability to perform full weight-bearing stepping on a treadmill in cats after a complete spinal cord transection (de Leon et al. 1998; Edgerton et al. 1997), which has supported the presence in the lower spinal cord of a central pattern generator (CPG) able to generate rhythmic motor activities in the absence of supraspinal descending inputs (Grillner et al. 1985). How the CPG can shape its functional properties in response to the training is not clear, but it is thought that reinforcement by sensory afferents of existing sensorimotor pathways, rather than generation of new connections, might be responsible for the beneficial effects (de Leon et al. 1998, 1999, 2001). There could also be anatomically altered synaptic connections, increased active zones of synapses, altered sensitivities of neurotransmitter receptors, or altered production of neurotransmitters. Sensory input provided during locomotor training are critical for driving the plasticity that mediates locomotor recovery and pharmacological treatments can be used to excite the spinal neurons that generate stepping (Edgerton 2001). So bicycle training along with baclofen (GABA_b agonist) can show a drastic improvement in locomotor pattern of SCI subjects as the paddling movement of the bicycle gives a constant sensory input by activating the receptors in the joints, muscles and tendons in the ankle, knee and hip joint of the hind limbs.

Locomotor training is beneficial in maintaining and even improving neural function following insult or disease (Wernig et al. 1999). It is recognized that trophic factors such as neurotransmitters are critical modifiers of the structure and function of neural networks such as 5-HT. Serotonin is a neurotransmitter of descending pathways from brain to spinal cord, primarily in the ventral and lateral funiculi of the spinal cord influencing interneurons and motor neurons via postsynaptic inhibition. The serotonergic cells of origin are in the raphe nuclei of the brain stem and the reticular formation. The serotonergic cells in the nucleus raphe obscurus and

nucleus raphe pallidus project to the intermediolateral cell column and ventral horn of the spinal cord. After complete or incomplete SCI, the descending 5-HT bulbospinal tracts in the lateral and anterior funiculi undergo a slow wallerian degeneration because of the small fiber size and lack of myelin, and this involves the axon terminals with the reduction of uptake, with the development of spastic paraparesis. The motor scores have been significantly correlated with changes in 5-HT staining in the ventral horn but not in the dorsal horn (Shapiro 1997). The 5-HT system surrounds the corticospinal tract in the lateral funiculus, which explains the correlation between losses of 5-HT and motor deficit after SCI. So an increase in the 5-HT in the lumbar spinal cord after SCI with baclofen and locomotor training can correlate with the improvement in the motor functions.

Physical activity even in an intact brain and spinal cord can induce the expression of trophic factors in the hippocampus and other brain regions (Gomez-Pinilla et al. 1997; Gomez-Pinilla et al. 1998), while exercise leads to the expression of trophic factors such as BDNF and NT-3 specific neural networks (Gomez-Pinilla et al. 2002, Ying et al. 2003) which are important in growth and neural function of the neurons. Exercise has been shown to induce BDNF in the lumbar enlargements of the uninjured spinal cord (Gomez-Pinilla et al. 2001) and treadmill walking increased labeling of BDNF, its receptor, trkB, and neurotrophin-4. Neurotrophin modulation induced by neuromuscular activity can play a role in facilitating functional recovery following SCI. The injured spinal cord generally loses the ability to synchronize and interpret the coordinated ensemble of afferent information that produces a predictable motor outcome in an uninjured patient and so produces random motor pool activation. This deficit may be due to the absence or rare occurrence of synchronized events normally associated with load-bearing stepping. In the absence of these coordinating events, the spinal cord loses the ability to synchronize input into functional movements of the limbs. A patient who is hypo responsive to

sensory input is less likely to respond to the proprioceptive input associated with load-bearing stepping, so the presence of spasticity, which is exaggerated stretch reflex due to hyper responsive sensory input, is a positive sign of the potential for a SCI patient to regain some locomotor ability. Clearly, understanding the physiological mechanisms that underlie spasticity will enhance our efforts to facilitate locomotor recovery in SCI patients. Activity-dependent motor training facilitates the recovery of posture and locomotion after a complete SCI in mammals. Because functionally recovered spinal animals i.e., SCI animals showed no evidence of regeneration of descending pathways (Joynes et al. 1999) or showed minimal changes in hind limb skeletal muscle properties (Roy et al. 1998, 1999) to account for the recovery characteristics, the functional behavior exhibited by these animals must have been mediated by the plasticity in existing spinal pathways. It is generally accepted in the literature that exogenous application of BDNF and NT-3 on spinal motoneurons improves the regeneration of the fibers in the spinal cord and thus can improve the outcome. Neurotrophins supplied by endogenous sources may have an even greater effect on compromised cells and motor training have shown to improve the levels of neurotrophins in the injured spinal cord. The positive effects of motor training have been documented in animals (Hodgson et al. 1994; de Leon et al. 1998) and humans (Harkema et al.1997). The success of rehabilitative strategies is highly task specific, which closely simulate the functional situation of walking are the most effective in promoting the restoration of locomotion (Edgerton et al. 1997; de Leon et al. 1998.) Rehabilitative strategies that stimulate walking, like treadmill or bicycling, are effective in improving locomotion in SCI due to the phasic sensory input produced by repetitive foot contact with the ground or the foot pad in the case of bicycle to result in the induction of activity dependent events such as increased neurotrophins levels in circuitry by repetitive loading of the hind limb (Gomez-Pinilla et al. 2005).

In summary, the above literature review shows that locomotor training whether it is voluntary, wheel running or treadmill prevents the development of spasticity by modifying the neurotransmitter levels and enforcing neuroplasticity in the surviving sensorimotor neurons so as to develop a rhythmic reflex pattern that leads to improvement in the gait. Also baclofen therapy started acutely can prevent the development of PICs which may be another cause of the late onset spasticity and reinduce the presynaptic inhibition by acting on the GABA_b receptors and upregulating them.

CHAPTER 3
EXPERIMENTAL DESIGN AND THE STANDARD METHODOLOGY USED IN THE
PROJECT

Objective

The whole thesis work was conducted in a rodent animal model using established techniques and protocols. Standard contusion SCI animal model was used in this project and the injury device and the animals used are described below. The experimental set up for spasticity measurement (Ankle torque and EMG), for locomotor gait assessment using footprints, and the open field locomotor (BBB) scoring are all standard techniques. The bicycle training methodology was developed in this lab and will be described in more detail. The following segments will describe those techniques and protocols used in this project (Figure 3-1).

Animal Subject

Twenty four twelve week old female Sprague-Dawley rats (SPF) weighing 220-260 g (Charles River Laboratories) at the start of this study were used in this project. Rats were housed two per cage, in 12 hour light/dark cycle, and given food ad libitum. The total number represents here is the actual experimental animals (24) not including the inadvertent losses: a) animals that died during surgery (2 animals), b) those excluded based upon post-injury selection criteria, (9 animals) or c) animals that died unaccountably during the 2 month training and drug therapy program (none). The total number of animals used was thirty five. An attending veterinarian supervised care of these animals. All procedures were performed under the guidelines of Animal Care and Use Committee.

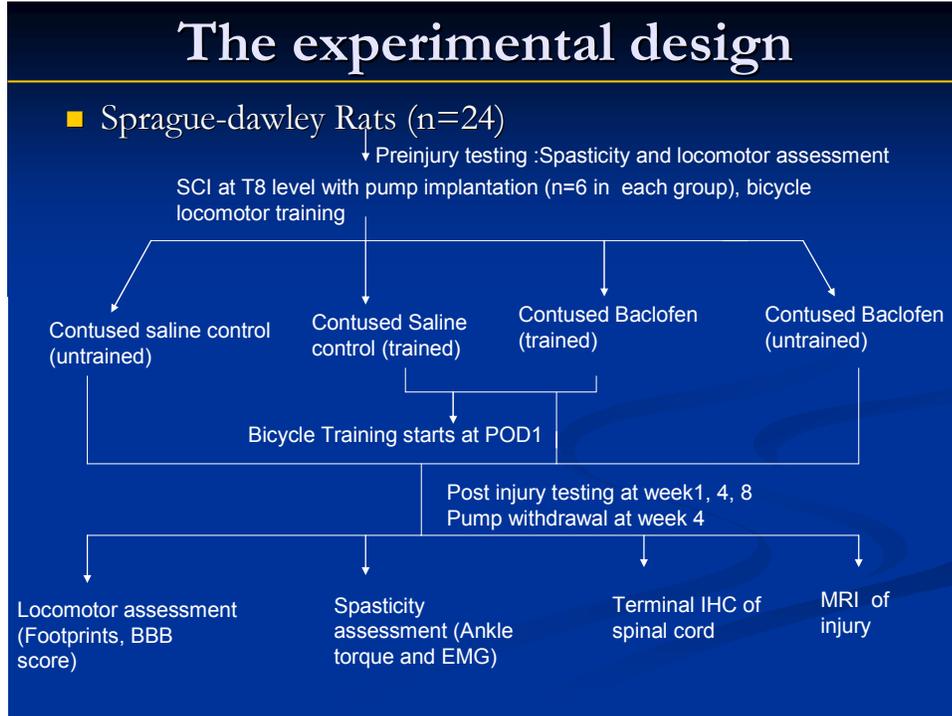


Figure 3-1 The experimental set up and procedures done in chart form

Contusion Injuries

Contusion injuries were produced using a standard New York University (NYU) Multicenter Animal Spinal Cord Injury Studies (MASCIS) impactor (Basso et al. 1996). The injury was performed under ketamine (100 mg/kg), xylazine (10 mg/kg, 1:3 with normal saline), and glycopyrrolate (200 µl in each animal) anesthesia and previously reported in detail (Bose et al. 2002, Thompson et al. 1992,1998). A laminectomy was performed at T8 segment, exposing the underlying dura. The spinal column was stabilized with angled Allis clamps on the T₇ and T₉ spinous processes. An incomplete spinal contusion was made at the T₈ segment of the spinal cord using the impounder tip of the MASCIS 10-g weight impactor device (2.4 mm in diameter) dropped from a height of 12.5 mm (computerized operation). The whole procedure was performed under aseptic conditions. The animals were monitored routinely and were given

postoperative care on a regular basis or as required until full bladder function was re-established and no evidence of pain or other discomfort was detected.

Baclofen Pump Implantation

All rats were anesthetized with subcutaneous injection of xylazine (6.7 mg/kg) followed by intraperitoneal ketamine (100 mg/kg)/glycopyrrolate (40 mg) (Reier et al. 1992; Thompson et al. 1992, 1993). Prior to implant, osmotic pumps (model 2004, 0.25 mL/h; Azla Corp., Palo Alto, CA) were filled with baclofen (3.2 µg/µL; implanted in twelve animals) or 0.9% physiological saline (implanted in twelve animals). Osmotic pumps were incubated overnight in 0.9% saline at 37°C in sterile conditions for immediate delivery of drug or vehicle. The dose rate was 0.8 µg/hr. Particular efforts were taken to avoid introducing air space within the infusion catheter. A 4cm vertical skin incision was made spanning the thoraco-lumbar juncture. A subcutaneous pocket was dissected to accommodate the osmotic pump. Musculature covering spinal processes was retracted from T₁₂-L₁, and a T₁₃-L₁ laminectomy was performed. The osmotic pump was maneuvered into the subcutaneous pocket and secured to surrounding musculature using 6.0 silk sutures. The infusion catheter was tunneled through the rostral bank of dissected musculature, and secured by polyacrylamide adhesion (Vetbond™ tissue adhesive; 3M, St. Paul, MN) to exposed spinal process. The wound margins of muscle and skin were infiltrated with a long-lasting local anesthetic (lidocaine). With the aid of a dissecting microscope, the duramater was cut and the silastic tubing inserted into the subarachnoid space of the lumbar enlargement. Excess tubing was secured to surrounding musculature with 6.0 silk sutures. Muscle layers were closed with absorbable sutures (Dexon II; Sherwood, Davis & Geck, Wayne, NJ) and the skin closed using stainless steel wound clips (Autoclip®; Becton Dickenson, Sparks, MD). Postoperative care included a 4mL subcutaneous injection of warmed (37°C) 0.9% physiological saline and overnight assistance with body temperature regulation using a temperature controlled

heating pad. The day after surgery behavioral and neurophysiological assessment were started (Figure 3-1). After 4 weeks of ITB treatment, the pump was removed by similar procedures, and the infusion catheter was ligated with 6.0 silk sutures and left in situ.

Bicycle Training



Figure 3-2 Motorized bicycle used for training the rats.

Careful attention was taken during training, as it was started acutely (PO day 1) after the injury. The animals were trained over the course of 2 months. The training schedule was performed 5 days a week using two 20 minute trials/day, starting from PO day 1. On the first day of training, the rats were given five minutes of training. The bicycle exercise regimen (Figure 3-2) involved immobilizing the rats in a custom made harness with the hind limbs suspended. The hind feet were strapped onto the pedals using cotton tapes. The exercise consisted of a pedaling motion, which fixed one limb while extending the other without overstretching the limbs. The cycling speed was 31 rotations/minutes (around 11 meters/min, distance wise). During the first week of training, the rat tail was attached to the aluminum support boom by surgical tape to

maintain the trunk stability during exercise. However, following second week of training, gradually the load on the pedals was increased by positioning the body harness towards the chest, so that the hind portion of the body was more extended over the pedal. The basic mechanism and principle of bicycle training is that of locomotor activity-initiated sensory input derived from weight bearing, as well as from the receptors of the skin, bones and muscles of the ankle, knee and hip joints of the hind limbs (Figure 3-2).

Footprints

To document hind limb gait abnormalities, footprints were acquired while rats walked at 11 m/min, along a 20 x 40 cm surface of a treadmill (Columbus Instruments, OH, USA). Prior to each trial run, hind limb footpads were coated with nontoxic blotter ink and the treadmill lined with recording paper. Footprints were acquired from each animal with six consecutive steps considered optimal for data analysis. Hind limb axis was measured using the angle of intersection between left and right hind foot axis during coordinated stepping. Foot axis was determined by a line passing through the third distal phalanges, metatarsophalangeal joints, and between the cuboid bone and medial cuneiform bone. The base of support was determined by measuring the horizontal distance between the central footpads of the hind feet. Prior to footprint recordings, the animals walked for a few minutes to accommodate to and become familiar with the treadmill walkway. Prior to the surgery preoperative footprints were taken to compare the changes in the walking pattern after injury and after training (Figure 3-2).

BBB Score

An open field-testing procedure (Basso, Beattie, and Bresnahan (BBB) 21-point scale) (Basso et al. 1995) was applied after the contusion injury at different time points to assess the locomotor deficit and its improvement following exercise and or baclofen therapy. These observations were made at POD 1, wks 4 and 8 in a blind fashion (Figure 3-2). The rats were

placed in a clean molded-plastic circular enclosure to walk freely to perform this procedure. It rates behavior from individual joint movements of the hind limb, to plantar stepping, to coordinate walking and finally the more subtle behaviors of locomotion, such as paw position, trunk stability and tail position. Observations are recorded for 4 min in an open field and then converted to a numerical score from the scale.

BBB Score Inclusion Criteria: to decrease injury variability of animals with in the study, the animals were scored using the BBB, at PO day 1 after injury. Those animals that scored > 7 were considered too mildly injured and were excluded from the study.

Statistical analyses of the data (velocity-dependent ankle torque, footprints and open field locomotor recovery) included between groups ANOVA and post hoc tests to achieve group differences.

Ankle Torque

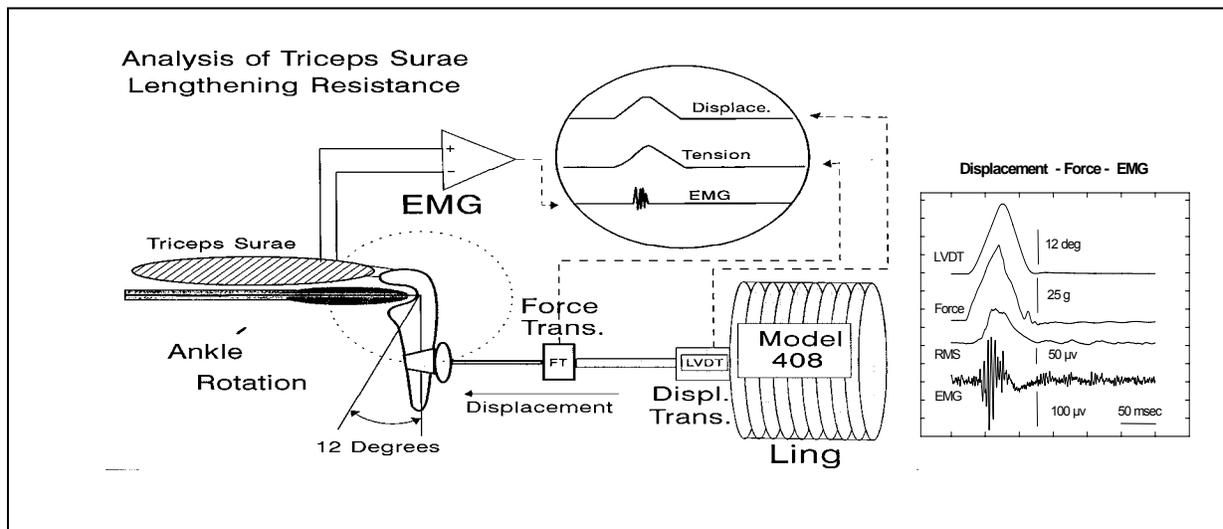


Figure 3-3 Ankle torque, displacement and EMGs are simultaneously recorded and time-locked to onset of dorsiflexion.

Pre-operative as well as postoperative recordings utilizing the ankle torques protocol were taken. Details regarding instrumentation, animal set-up and recording procedures have been

previously reported (Bose et al. 2002; Thompson et al. 1996) but will be described below briefly. Rats were immobilized in a custom designed trunk restraint, without trauma or apparent agitation. All recordings were performed in awake animals. The proximal portion of the hind limbs to the mid-shank, were secured in a foam fitted cast that immobilized the limb while permitting normal range of ankle rotation (60 to 160 degrees). The lengthening resistance of the triceps surae muscles was measured indirectly by quantitating ankle torque during 12-degree dorsiflexion rotations of the ankle from 95 through 83 degrees. Contact with the foot was achieved using a foam-fitted cradle aligned with the dorsal edge of the central footpad 2.6 cm distal to the ankle joint. The angle of contact between the displacement shaft and the moment arm was 95 degrees (Figure 3-3). The neural activity of the triceps surae muscle was measured using transcutaneous EMG electrodes. The electrode was inserted in a skin-fold over the distal soleus muscle just proximal to the aponeurotic convergence of the medial and lateral gastrocnemii into the tendonocalcaneus. A reference electrode was placed in a skin fold over the greater trochanter. A xylocaine 2% jelly (Lidocaine HCl, Astra USA Inc.) was applied over the electrode insertion points to minimize pain during recording. A topical antibiotic ointment (a combination of Bacitracin, Neomycin and Polymyxin B; Fougere Altana Inc., Atlanta, GA) was also applied on these areas after taking off the electrodes at the end of each trail to reduce any chance of infection. Controlled dorsiflexion was achieved through the use of an electromechanical shaker (model 405, Ling Dynamic systems, Royston Herts, U.K.). A force transducer (LVDT) (model FT-03; Grass Instruments, Quincy, MA) was placed in series between the output shaft of the ling shaker and the central footpad (figure 3.3). Root mean square (RMS, i.e., a 0.707-DC equivalent of the full wave rectified AC signals) of EMG bursts was also recorded on an additional channel of the signal acquisition system. EMG magnitudes are

reported as mean RMS magnitude of the EMG bursts time-locked to ankle dorsiflexion. Collectively, this arrangement allows for simultaneous monitoring of triceps surae EMG, resistive force, and velocity of shaft displacement. Recorded data were processed using Data Wave Technologies signal acquisition system (model 32C; Data Wave Technologies, Denver, CO) with an analogue to digital sampling rate of 200 KHz. Dorsiflexions of 12 degrees were performed with 3-sec intervals at 49, 136, 204, 272, 350, 408, 490, and 612 deg/sec. At each test velocity, five consecutive sets of waveforms, 5 waveforms per set, that is, 25 in total, were recorded, signal averaged, and saved for subsequent analysis. An average of two time points recording was used for data analysis. Regression of ankle torque against the ankle joint rotation yielded resistance (torque) per degree of rotation for each velocity variable of the protocol with torque values expressed in Kdynes (1000 dynes = 1 Kdyne). The protocols were performed using the fastest rotation first, then the slowest rotations

Immunocytochemistry of Spinal Cord Tissue

The standard Fluorescent and Avidine-Biotine Complex (ABC) immunohistochemical techniques were utilized for visualizing neurotransmitters such as GABA_b, 5-HT, GAD₆₇, BDNF, GAP43. These techniques allow the visualization of varicosities as well as fibers that are interpreted as axons using 40µm thick cryostat sections of the lumbar and thoracic spinal cord. Qualitative evaluation was done using a light microscope and bright field microscope.

In brief, spinal cord segments (thoracic segments caudal to the injuries, and lumbar spinal cord, L₃-L₆) were dissected and removed after perfusion (4% paraformaldehyde in PBS) and kept in the same fresh fixative mixture for 1 hour and was cryoprotected for at least 2 days in 30% sucrose in 0.1 mol PB. The specimens were cut serially (cross section) by cryostat (40 µm

thickness) and processed by Avidine-Biotine Complex (ABC) and Florescent immunohistochemistry (IHC).

The immunoreactivity of GAD₆₇, GABA_b, GAP₄₃ and BDNF were identified in lumbar and thoracic spinal cord. The cryostat cut sections were incubated with primary antibodies generated against GAD₆₇ (mouse mAb; 1:1,000, National Hybridoma Laboratory, St Luis, USA), GABA_b (guinea pig mAb, 1:4,000; Chemicon International), GAP₄₃ (mouse mAb, 1:5000; Chemicon International), and BDNF (rabbit Ab, Chemicon International) for 24-48 h at 4° C. The sections were then washed in PBS and incubated for 1.5 h in alexa fluor-congugated appropriate anti-mouse, anti-guinea pig or anti-rabbit IgG (1:1000, Molecular Probes). For ABC technique, anti-guinea pig (1:200; Chemicon), anti-mouse, and anti-rabbit (1:200; mouse and rabbit Elite kits, Vector Lab) secondary antibodies were used to bind with appropriate primary antibodies. Sections were then washed again and mounted for microscopic analyses.

CHAPTER 4 RESULT

The following sections present results of velocity-dependent lengthening resistance due to dorsiflexion of the ankle, open-field locomotion ability and gait (footprints) analyses.

Velocity-Dependent Ankle Torque and Associated EMG's

Pre-injury Ankle Torque and EMG Data

Baseline measures of velocity-dependent ankle torques and extensor EMG's were obtained from all animals before injury at post-injury weeks 1, 4 and 8.

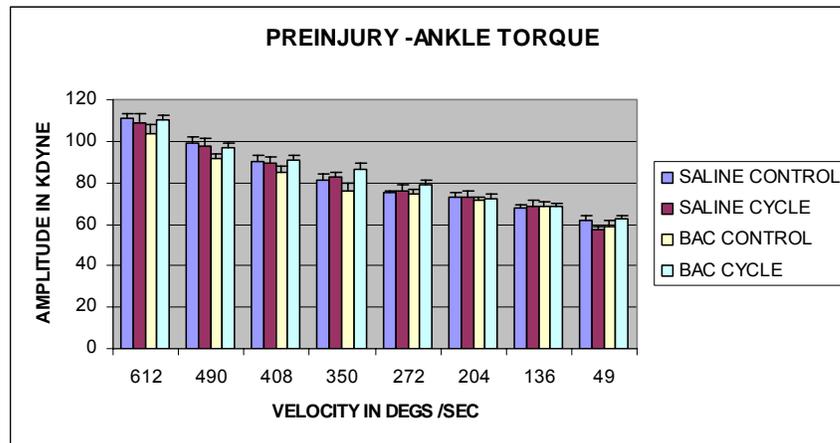


Figure 4-1 Pre-injury ankle torque graph.

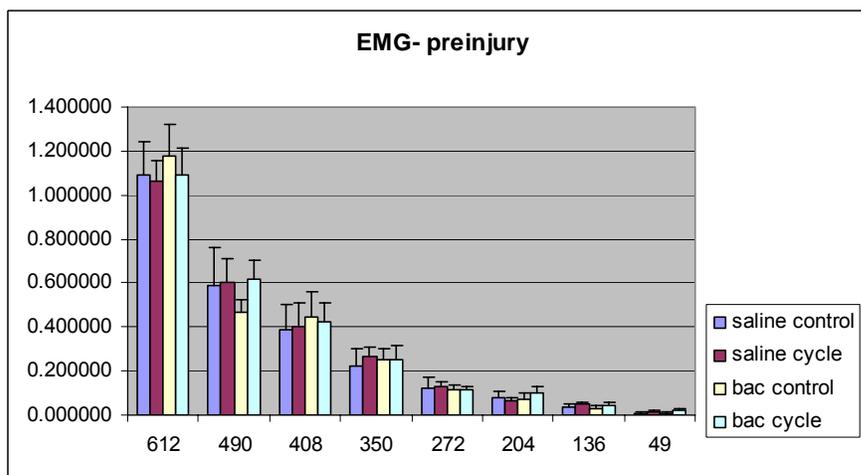


Figure 4-2 Pre-injury EMG graph.

Postoperative Week 1 Ankle Torque and EMG Data

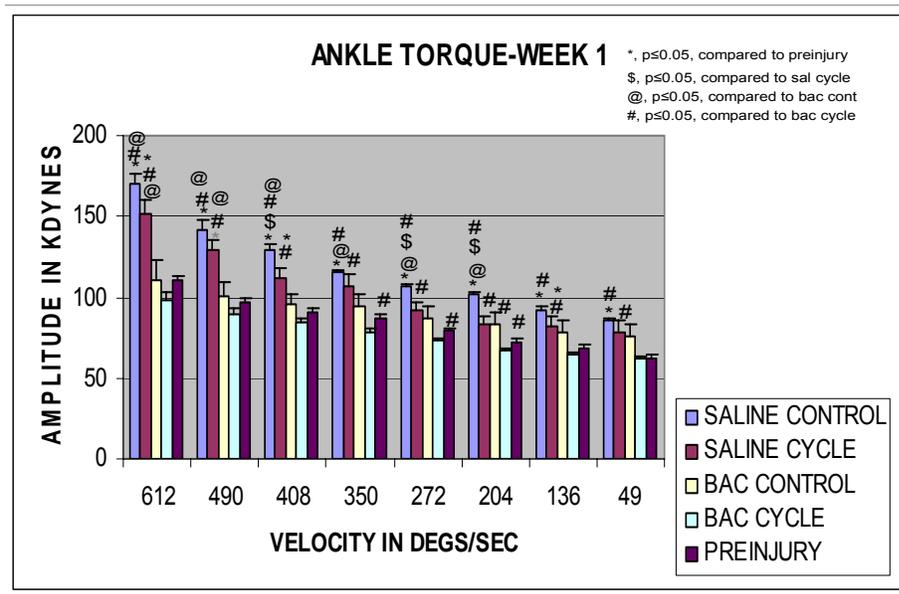


Figure 4-3 Ankle torque graph at post-op week 1 with significant differences.

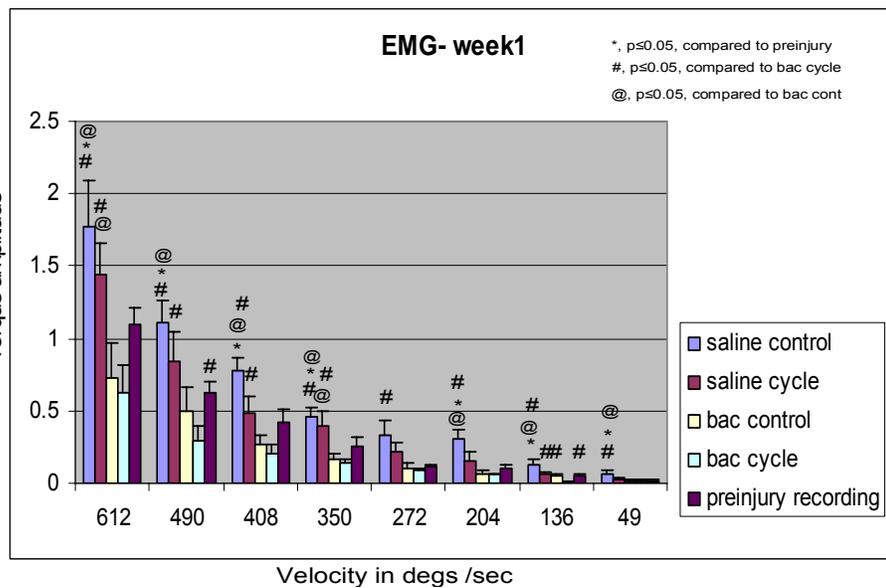


Figure 4-4 Electromyeloencephalogram graph at post-op week 1 with significant differences.

When tested at one week following injury, baclofen cycle group revealed significantly decreased magnitudes of ankle torque during rotation at each of the eight ankle rotation

velocities, even less than the control values recorded before injury (Figure 4-3). These were about 11% lower at the highest velocity (612 deg/sec) and 0.8% lower at the lowest velocity (49 deg/sec). Similarly the mean values observed for the baclofen control group did not show significant changes compared to the pre-injury group, only 7% increase at the highest test velocity (612 deg/sec), and 28% increase at the lowest test velocity (49 deg/sec) (Table 4-1).

Mean values observed for the saline control group revealed significantly increased magnitudes of ankle torque during rotation at each of the eight ankle rotation velocities when compared to corresponding pre-injury values (Figure 4-3). Approximately a 52% increase was observed at the highest velocity (612 deg/sec), and a 38% increase at the lowest test velocity (49 deg/sec) (Table 4-1). These values were significantly different when compared to comparable measures in both baclofen cycle and baclofen control groups (Figure 4-3).

The saline cycle group also showed a significant increase in magnitude of ankle torque at each of the eight ankle rotation velocities tested when compared to pre-injury values (Figure 4-3), approximately 39% increase at the highest test velocity (612 deg/sec) and 37% increase at the lowest test velocity (49 deg/sec) (Table 4-1).

There were significant differences in the velocity-dependent ankle torques between saline control and saline cycle groups when compared to corresponding values obtained from baclofen control, baclofen cycle and pre-injury groups; but no significant differences were observed between baclofen control, baclofen cycle or pre-injury groups (Figure 4-3).

The EMG-RMS magnitudes recorded at each of the test velocities closely paralleled the velocity dependent ankle torque measurements (Figure 4-4). Significant parallel increases in ankle torque and EMG magnitude, respectively, were observed during ankle rotations at the

slowest four velocities at post-op week 1 in control and saline cycle group when compared to baclofen cycle group

Table 4-1 Ankle torque week 1- percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk1 Ankle Torque Data % ↑↓							
	612	490	408	350	272	204	136	49
Saline Control	52%↑	43%↑	43%↑	41%↑	41%↑	39%↑	35%↑	38%↑
Baclofen Cycle	11%↓	7%↓	7%↓	8%↓	7%↓	8%↓	6%↓	0.8%↓
Saline Cycle	39%↑	31%↑	25%↑	29%↑	20%↑	14%↑	19%↑	37%↑
Baclofen Control	7%↑	10%↑	12%↑	24%↑	18%↑	17%↑	14%↑	28%↑

Table 4-2 Electromyeloencephalogram week 1 - percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk1 EMG Data % ↑↓							
	612	490	408	350	272	204	136	49
Saline Control	62%↑	89%↑	101%↑	108%↑	168%↑	289%↑	289%↑	695%↑
Baclofen Cycle	42%↓	52%↓	50%↓	45%↓	22%↓	39%↓	67%↓	8%↓
Saline Cycle	35%↑	40%↑	20%↑	49%↑	73%↑	143%↑	29%↑	109%↑
Baclofen Control	38%↓	5%↑	41%↓	34%↓	6%↓	11%↓	50%↑	103%↑

Postoperative Week 4 Ankle Torque and EMG Data

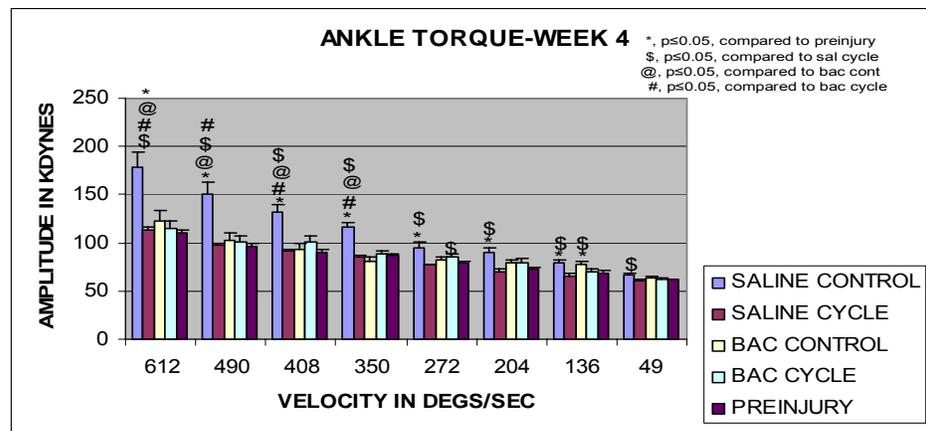


Figure 4-5 Ankle torque graph at post-op week 4 with significant differences.

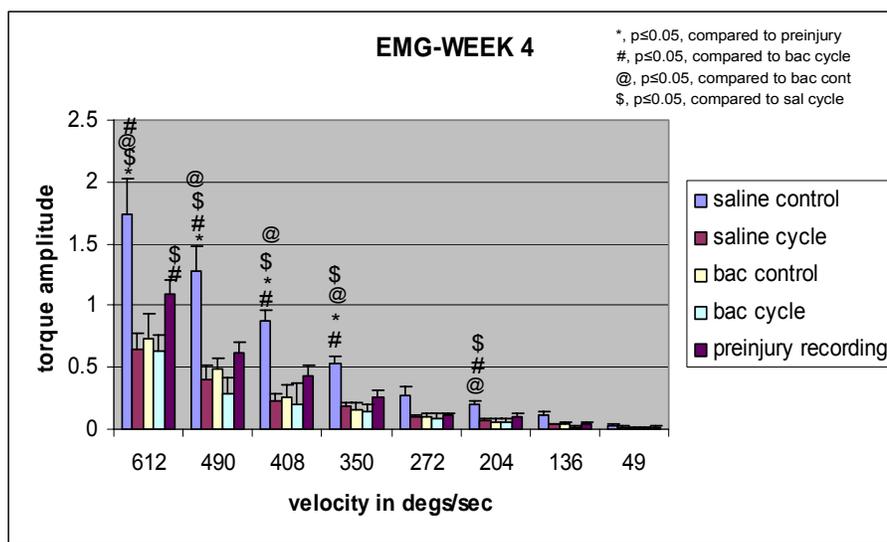


Figure 4-6 Electromyeloencephalogram graph at post-op week 4 with significant differences.

At post-injury week 4, a significant decrease in the ankle torques was observed at all test velocities in the saline cycle contused group compared to these values observed at week 1 (Figure 4-5) for example, at the highest velocity (612 deg/sec), at week 1 the ankle torques were 39% greater than control, but only 4% greater at week 4; a 35% decrease. And at the lowest test velocity (49 deg/sec), the week-4 saline cycle animals revealed ankle-torques that were 5% greater than controls, compared with 14 % at week 1 for these measures. On the other hand, baclofen control and baclofen cycle showed the same pattern as week 1. The saline control group ankle torques remained significantly elevated, and were similar to week 1 data, a 61% increase at highest test velocity (612 deg/sec) and a 9% increase at the lowest velocity (49 deg/sec) (Table 4-3). The ankle torque magnitude showed the same pattern in the lower velocities as that of higher velocities.

The EMG magnitudes mirrored the pattern of the ankle torques in all the four groups at all the velocities, there was no significant difference in the EMG magnitude at all the lower 4 velocities when compared to higher velocities in post operative week 4 (Figure 4-6) (Table 4-4).

Table 4-3 Ankle torque week 4- percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk 4 Ankle Torque Data % ↑↓							
	612	490	408	350	272	204	136	49
Saline Control	61%↑	52%↑	47%↑	43%↑	27%↑	22%↑	18%↑	9%↑
Baclofen Cycle	5%↑	5%↑	10%↑	2%↑	7%↑	9%↑	2%↑	0.05%↑
Saline Cycle	4%↑	0.2%↑	3%↑	3%↑	1%↑	5%↓	6%↓	5%↑
Baclofen Control	18%↑	13%↑	9%↑	6%↑	11%↑	12%↑	13%↑	8%↑

Table 4-4 Electromyeloencephalogram week 4- percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk 4 EMG Data % ↑↓							
	612	490	408	350	272	204	136	49
Saline Control	60%↑	117%↑	127%↑	138%↑	122%↑	152%↑	248%↑	296%↑
Baclofen Cycle	27%↓	27%↓	8%↑	15%↑	49%↑	20%↑	14%↓	33%↓
Saline Cycle	39%↓	33%↓	41%↓	28%↓	23%↓	3%↑	16%↓	43%↑
Baclofen Control	27%↓	7%↓	33%↓	17%↓	31%↑	13%↑	21%↑	128%↑

Postoperative Week 8 Ankle Torque Data and EMG Data

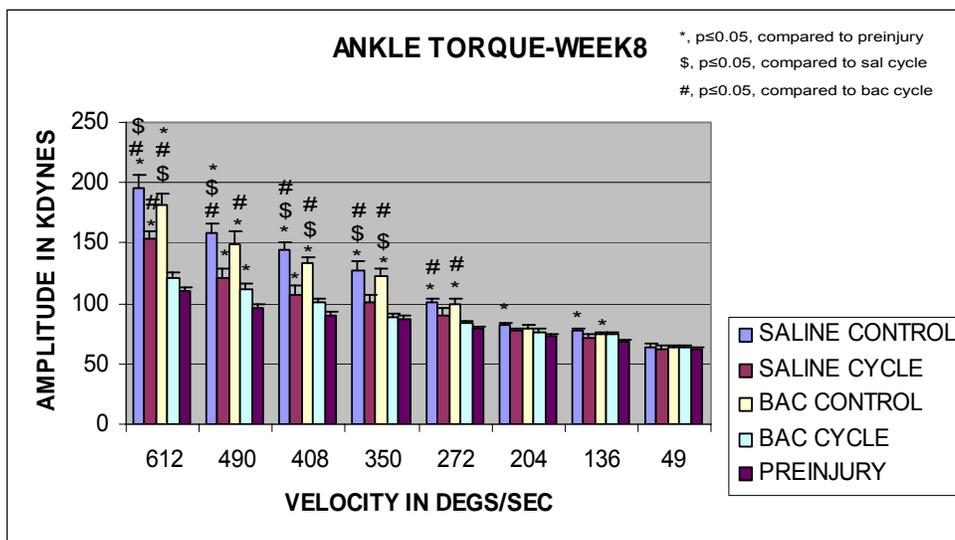


Figure 4-7 Ankle torque graph at post-op week 8 with significant differences.

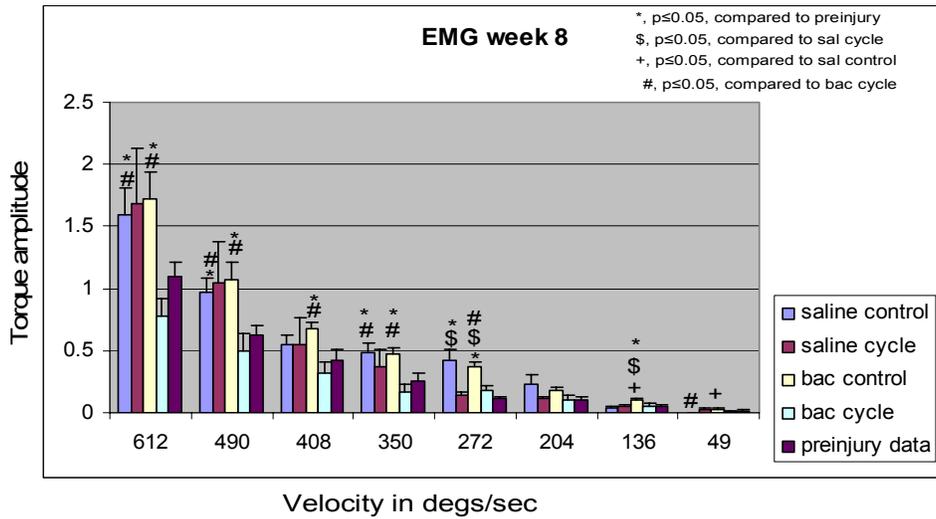


Figure 4-8 Electromyoelecephalogram graph at post-op week 8 with significant differences.

By post-op week 8, significant velocity-dependent ankle extensor spasticity re-appeared in the baclofen untrained contused group, a 75% increase at the highest velocity (612 deg/sec) compared to the corresponding value of pre-injury control group (Table 4-5). While only an 8% increase was observed at the lowest test velocity (49 deg/sec) (Figure 4-8). The velocity dependent ankle torques in saline control group appeared similar to those observed at post-op week 1 and 4, suggesting that this significant velocity dependent increase in ankle torque was enduring. Surprisingly, at this post-injury time point, this re-emergent spasticity was not observed in baclofen cycle group; only a 10% increase in ankle torque was observed at the highest test velocity (612 deg/sec) and only a 1% increase was observed at the lowest velocity (49 deg/sec), even after removal of the baclofen pump. The saline cycle group showed a 41% increase in magnitude in ankle torques at the highest velocity (612 deg/sec) and a 9% increase at the lowest velocity (49 deg/sec), relative to these measures in the pre-injury control group. At this point, EMG magnitudes were also observed to be increased significantly at the two fastest

ankle rotation velocities (490 and 612 deg/sec) in both saline and baclofen control and saline cycle group as compared with the baclofen trained contused animals. Moreover, the EMG magnitudes were observed to be decreased for the saline cycle group at the next 3 test velocities (408-272 degs/sec) (Figure 4-8). No significant increases in ankle torque or EMG magnitude were observed during ankle rotations at the slowest four velocities at post contusion week 4 and post-op week 8. The EMG pattern for baclofen cycle remained similar to the ankle torque data for the same week i.e., not much increase in magnitude as compared to pre-injury data (Table 4-6).

Table 4-5 Ankle torque week 8 - Percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk 8 Ankle Torque % Data ↑↓							
	612	490	408	350	272	204	136	49
Baclofen Control	77%↑	60%↑	60%↑	55%↑	33%↑	12%↓	14%↑	4%↑
Baclofen Cycle	10%↑	15%↑	11%↑	2%↑	5%↑	6%↑	7%↑	1%↑
Saline Cycle	41%↑	24%↑	20%↑	22%↑	19%↑	5%↓	5%↓	9%↑
Baclofen Control	75%↑	63%↑	57%↑	61%↑	34%↑	12%↑	8%↓	8%↑

Table 4-6 Electromyeloencephalogram week 8 - Percentage (%) change (↑↓) compared to pre-injury.

Groups	Wk 8 EMG Data % ↑↓							
	612	490	408	350	272	204	136	49
Saline Control	46%↑	65%↑	42%↑	118%↑	243%↑	196%↑	12%↑	51%↓
Baclofen Cycle	29%↓	20%↓	24%↓	33%↓	53%↑	1%↓	19%↑	43%↓
Saline Cycle	58%↑	74%↑	36%↑	41%↑	15%↑	66%↑	17%↑	74%↑
Baclofen Control	46%↑	128%↑	52%↑	84%↑	228%↑	142%↑	216%↑	235%↑

Gait and Open Field Locomotion

Footprint Analysis: Gait

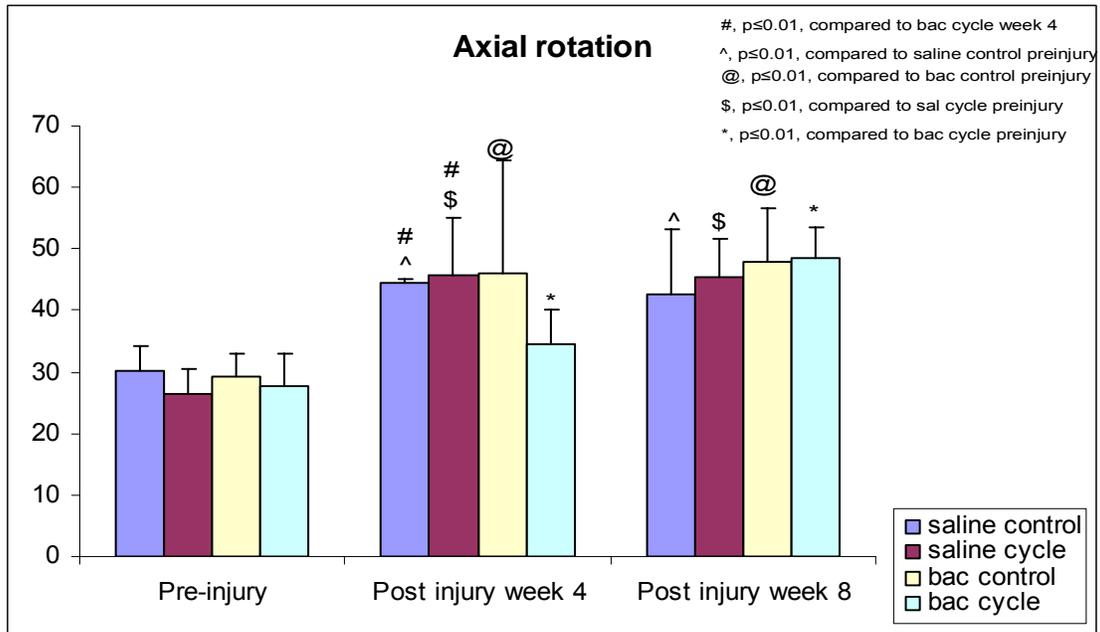


Figure 4-9 Axial rotation graph of all 4 groups at different time points with significant differences.

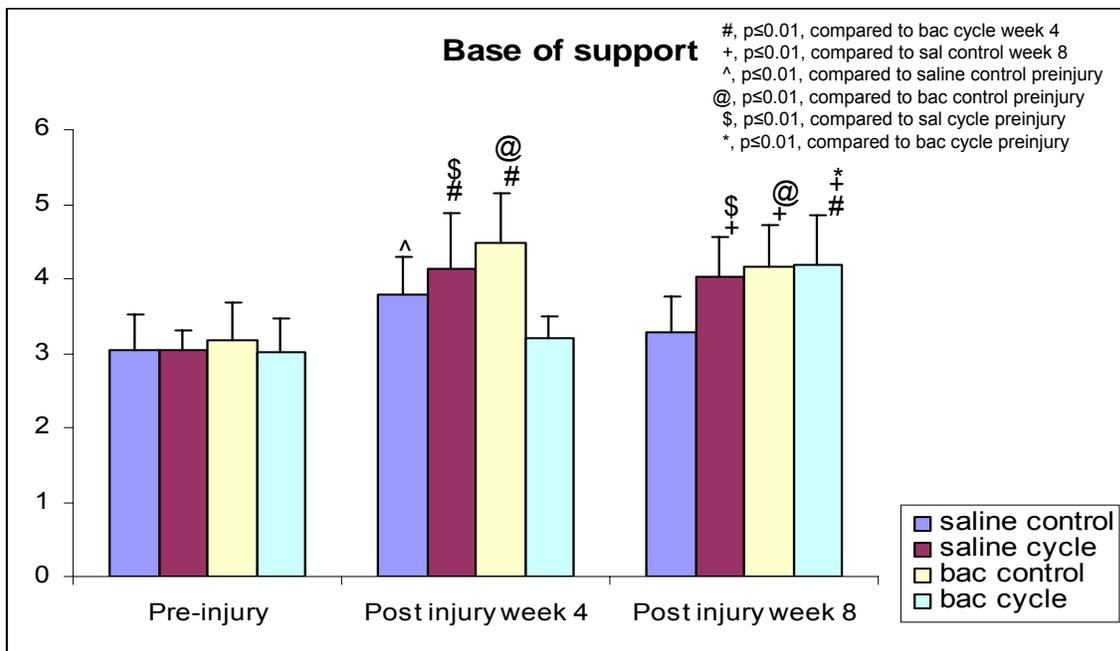


Figure 4-10 Base of support graph of all 4 groups at different time points with significant differences.

Footprint analyses were performed pre-injury, post-op week 4 and week 8. Before injury, limb axis and base of support were measured to be 28.33 ± 2.00 degs and 2.91 ± 0.37 cms, respectively. At post-op week 1, in saline control group the values for limb axis and base of support were 44.45 degs and 3.79 cms respectively, whereas in baclofen controls group the readings were 46.08 degs and 4.49 cms respectively. The baclofen training group showed the least value 34.44 degs and 3.21 cms, whereas the saline cycle group showed 45.86 degs and 4.13 cms respectively. Thus after 1 month post-op saline control and baclofen cycle showed the least change when compared to the pre-injury values (Figure 4-9 and 4-10). Compared with the pre-injury control values, these measures revealed that limb axis and base of support were significantly increased in the baclofen untrained contusion-injured animals and saline cycle group during week 4. At post-op week 8, the trend was reversed with baclofen cycle showing increase in the base of support and angle of axial rotation as compared to all the other 3 control groups at same time point and post-op week 4 and also compared to pre-injury data. At post-op week 8 ironically saline control showed the most improvement in all the 4 groups even without treatment (Figure 4-9 and 4-10).

Open Field Locomotor Recovery

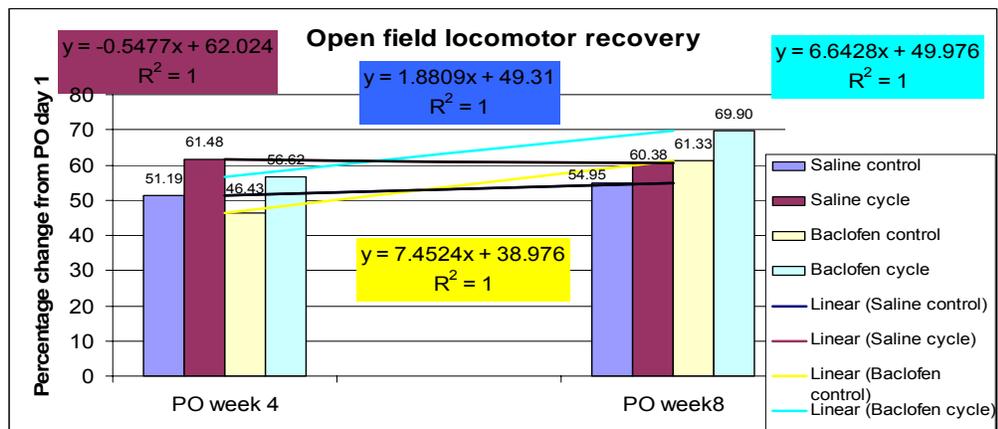


Figure 4-11 Graph of BBB showing percentage change in score at post-op week8 compared to post-op week 4.

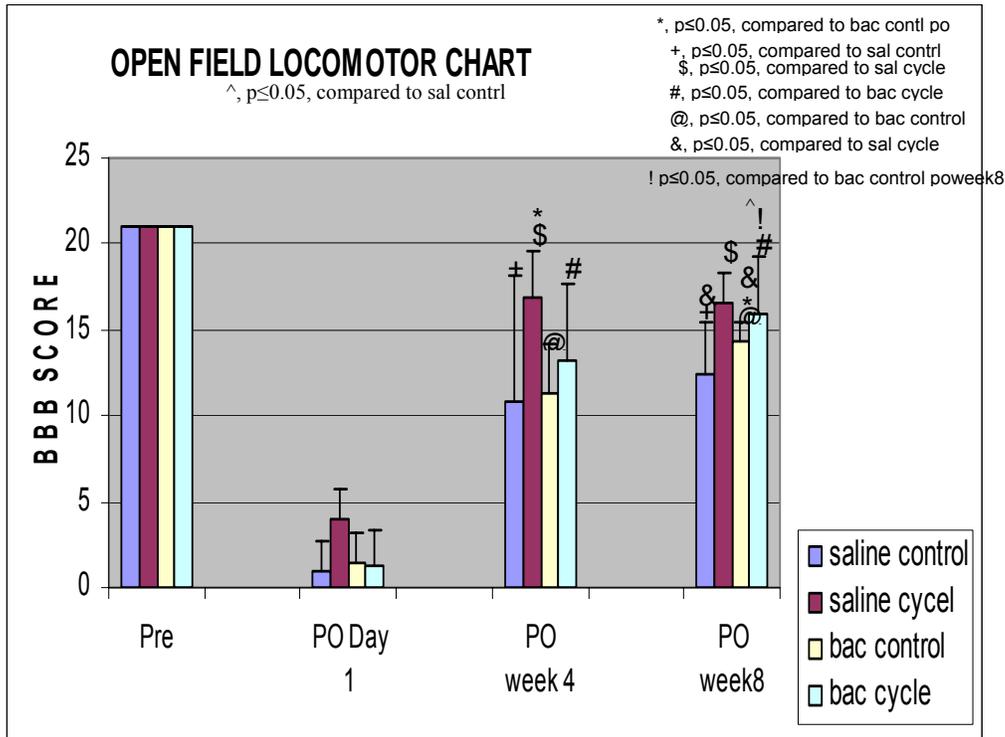


Figure 4-12 Open field locomotor graph showing BBB score of all 4 groups at different time points with significant differences.

Open field locomotor capacity (BBB) was scaled in both trained and untrained animals before injury, at post-op day 1, at post-op week 4, and at post-op week 8 to evaluate recovery during the early, intermediate, and late phases of recovery. Before the start of the treatment (bicycle and drug alone or combination), at postoperative day 1 the saline cycle group animals had the highest score as compared to the 3 other groups.

At post-op week 4, saline cycle trained contused animals displayed extensive movement of all three joints of the hind limb, (mean score, 17 ± 2) i.e., about 61% recovery compared to post-op day 1, but as a group, these data revealed significant variability in the scores among the groups (Figure 4-11). Baclofen cycle trained animals also showed good improvement in their score (mean, 13 ± 2) with a total recovery approximately a 56% increase from post-op day 1 (Figure 4-11). In contrast, baclofen control group (mean score 12 ± 1) showed about 46% recovery while saline control animals (mean score 11 ± 1) showed about 51% recovery when compared to post-op day 1 scores respectively (Figure 4-11 and 4-15). Bicycle trained animals

displayed a frequent to consistent weight supported plantar stepping and occasional to frequent FL-HL coordination (mean score, 15 ± 2). This BBB score in the bicycle trained group was significantly greater ($p < 0.05$, ANOVA) than values observed in either of the untrained groups (Figure 4-11).

At post-op week 8, baclofen cycle animals revealed scores that were significantly increased (mean score, 16 ± 1) when compared to values of the post-op week 4 and post-op day 1. This mean represented a recovery of approximately 70% when compared to post-op week 1 and 14% improvement compared to post-op week 4. Whereas, saline cycle group did not show any increase in its BBB score, instead revealed a drop (mean score, 16 ± 1) when compared to values of post-op week 4 score. In fact, its condition deteriorated by about 1.5% compared to post-op week 4 (Figure 4-11). Saline untrained animals showed increase in their score (mean score, 13 ± 2), about 15% improvement as compared to post-op week 4. Baclofen control group showed an increase in its score (mean score, 15 ± 0.2) i.e., about 15% improvement as compared to post-op week 4. Both of the BBB scores in the locomotor trained groups were significantly greater ($p < 0.05$, ANOVA) than the scores recorded for the untrained groups. However, at this stage, animals of both trained and untrained groups showed consistent FL-HL coordination and consistent weight supported stepping (mean scores, bicycle, 14.25 ± 1.4 , control, 15.25 ± 1.7). Please note, the terminologies never (0%), occasional (less than or equal to half, $\leq 50\%$), frequent (more than half but not always, 51-94%), and consistent (nearly always or always, 95-100%) used above (Basso et al. 1995)

The BBB score for an uninjured rat is 21 points.

In summary, open field locomotor recovery scores scaled at post-op weeks 4 and 8 were significantly higher in both of the training groups compared with untrained controls. The

baclofen cycle group demonstrated the highest recovery at post-op 2 month, which was also significantly higher than the untrained group. However, at post-op week 8, both baclofen and saline training groups showed similar recovery (ANOVA), whereas in post-op week 4, the saline cycle group showed more recovery than the baclofen cycle. The differences in the recovery may, in part, be reflected by the relative starting point; the saline cycle group started at a very high score compared to baclofen cycle group at post-op day 1.

Neurotransmitters

Immunocytochemistry results are shown in Figures 4-13 - 4-17.

GABA_b

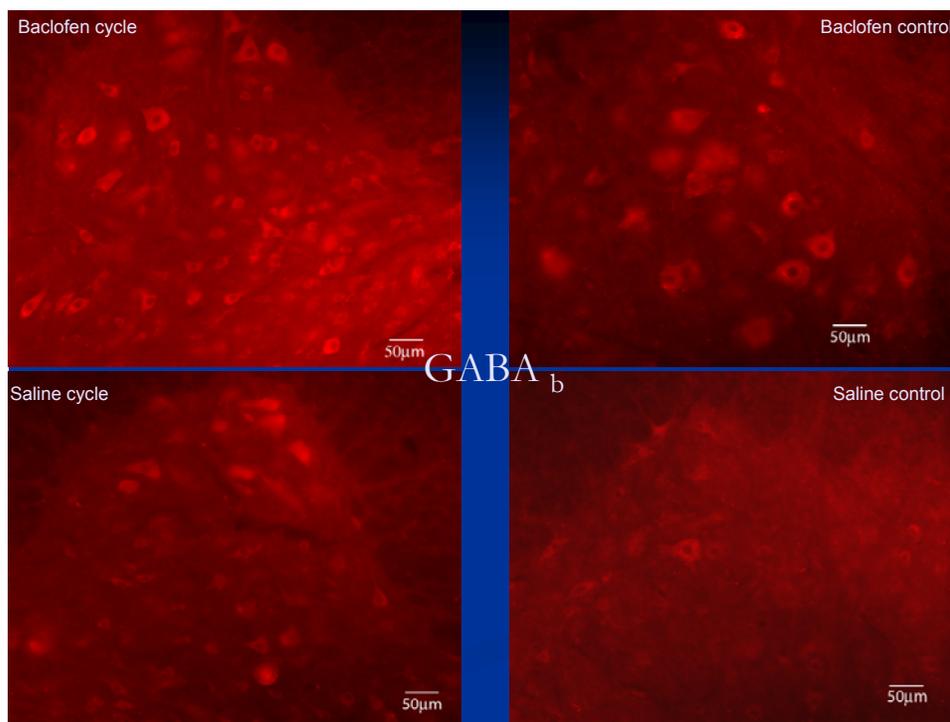


Figure 4-13 Immunohistochemistry of GABA_b receptors in lumbar spinal cord tissues.

In this Figure there is an increased expression of GABA_b receptors in the baclofen cycle as compared to the other three groups, with saline cycle group showing more expression than baclofen control and saline control showing the least expression

5-HT (Serotonin)

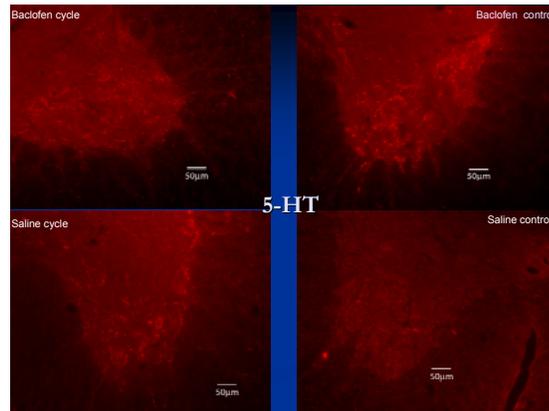


Figure 4-14 Immunohistochemistry of 5-HT (Serotonin) fibers in thoracic spinal cord tissues.

In this Figure there is an increased expression of 5-HT fibers in the baclofen cycle as compared to the other three groups, with baclofen control group expressing more fibers than saline cycle and saline control showing the least expression.

BDNF

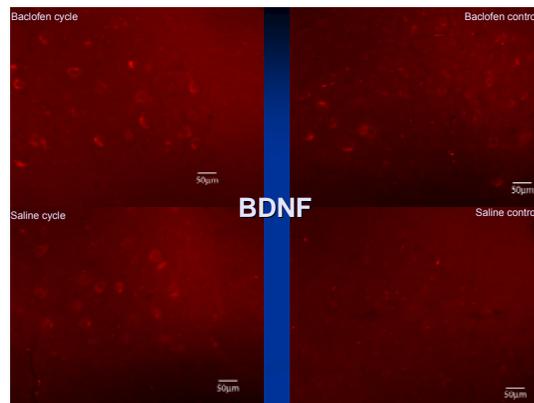


Figure 4-15 Immunohistochemistry of BDNF (Brain derived neurotropic factor) in lumbar spinal cord tissues.

In this Figure there is an increased expression of BDNF neuromolecules in Cycle Group's both baclofen and saline as compared to the baclofen control and saline control, which may point to the fact that exercise tends to increase the expression of BDNF.

GAP₄₃

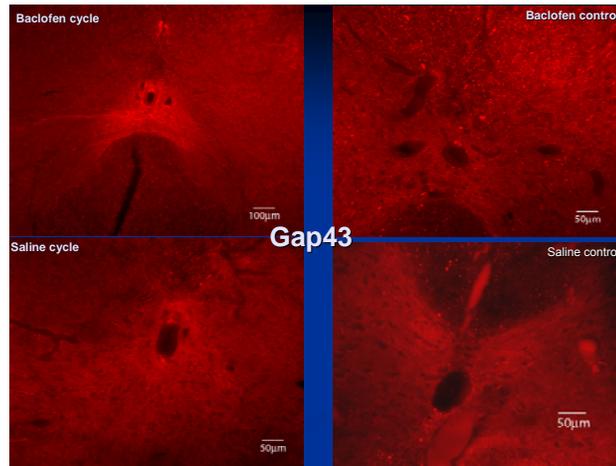


Figure 4-16 Immunohistochemistry of GAP₄₃ fibers in central canal of thoracic spinal cord tissues.

GAP₄₃ a neurotropic factor having functions similar to BDNF showed an increased expression in baclofen cycle and somewhat more in saline cycle then compared to baclofen control and saline control.

GAD₆₇

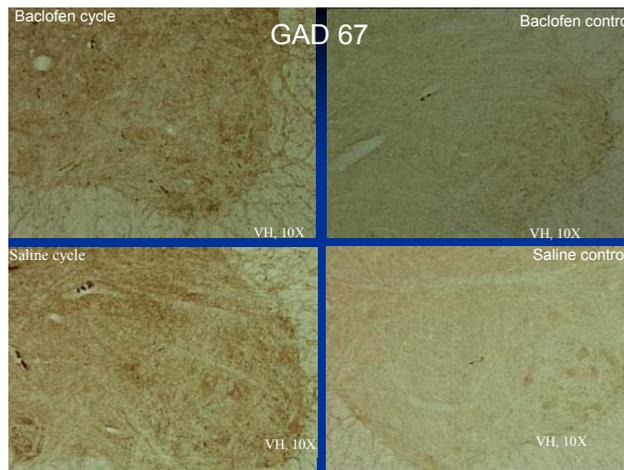


Figure 4-17 Avidine-Biotine Complex (ABC) figures of GAD₆₇ fibers in thoracic spinal cord tissues.

In this Figure GAD₆₇ showed an increased expression in baclofen cycle as compared to saline cycle and also increased expression in baclofen control, while saline control showed minimal expression.

Result Summary

The above results support the conclusion that baclofen cycle showed the greatest improvement with the lowest torque amplitude in all the velocities tested for ankle torque and EMG. The BBB score showed greatest percentage improvement in post-op week 8 compared to post-op day 1. Immunocytochemistry images showed an increased expression of the presynaptic inhibitor molecule GABA_b in baclofen cycle group as compared to all the other groups. Also there was an increased expression of neurotrophic factors such as BDNF, GAP₄₃, 5-HT and GAD₆₇, which is an indication of improvement in locomotion and increased growth of axonal fibers. Baclofen alone and locomotor treatment groups did show some improvement until post-op week 4 but later were not able to maintain the same pattern in post-op week 8. The saline control group did not show any improvement in spasticity at all time points but an improvement in BBB score which indicates that they have an intrinsic ability to initiate stepping without any treatment.

CHAPTER 5 DISCUSSION

The purpose of these studies was to test the hypothesis that acute treatments using baclofen and locomotor training, individually, and in combination, would significantly decrease the development of hyper reflexive spasticity following SCI by comparing velocity dependent ankle torque and time locked triceps surae EMG. Several behavioral tests (e.g. footprint analysis, and open field locomotor assessment) were also performed to test the influence of these treatments on aspects of locomotor functional outcome. In addition, to gain a better understanding of the fundamental neurobiology associated with SCI and the treatments, the expression of certain neurotransmitters that play an important role in spasticity and locomotion was compared in the treated and non-treated groups.

Compared with non-treated animals, individual treatment using locomotor training or ITB significantly reduced the development of early onset spasticity. However, ITB treatment during the first month post-injury did not decrease the development of late onset spasticity, while cycle training reduced the late onset spasticity by approximately 50%. The combination therapy (ITB and cycle training) profoundly reduced the development of both early and late onset spasticity.

Ankle Extensor Stretch Reflex

Saline Control

In saline control group there was hyper reflexive pattern (spasticity) seen at all the velocities tested and at all time periods (i.e., at post-op week 1, 4 and 8 with the EMG signals showing the same pattern as that of the ankle torque). This shows that when no treatment is given following SCI there is development of spasticity after the initial hypo reflexive period as the repetitive proprioceptive signals from the sensory receptors are not controlled and it leads to

spasm of the flexor muscle. So some form of treatment is essential for the treatment of spasticity following SCI.

Saline Cycle

The saline cycle group showed hyper reflexive pattern at post-op week 1. These data suggest that only exercise sufficiently addressed the mechanism that led to the hyper reflexia at this time point. These presumably include hyperactivity of the cells in the spinal cord after injury due to the release of the excitatory neuromolecules and the membrane damage which results in imbalance in the extracellular and intracellular components which is most common till week 1 post-injury. The EMG signals at this week also showed the same pattern.

In contrast, at post-op week 4, the ankle torque amplitude data of this group showed a pattern of hypo reflexia. This decrease in excitability may be due to the exhaustion of excitatory neurotransmitters after a certain time period after injury. Another possibility is that training induced activity may initiate a balancing of the neurotransmitters and preserve descending controls of segmental regulatory processes. As it has been reported in our lab studies, a pattern of hypo reflexia was observed at post-op week 2 in untrained and trained animals without drug treatment and continued up to week 6. Immediately after SCI motoneurons receive unusually large EPSPs from cutaneous stimulation consistent with the acute loss of descending brainstem innervations of the dorsal horn. These EPSPs do not easily cause reflexes immediately after injury, because of the profound loss of motoneuron excitability that occurs after injury from other mechanisms, such as decreased dendritic PICs. Recent studies indicate that a significant decrease in these postsynaptic, dendritic excitatory mechanisms may play an important role in the hypo reflexia at this time (Li et al. 2004), due to the loss of intrinsic persistent inward calcium and sodium currents (PICs) that normally prolong and amplify synaptic inputs. In addition, these investigators' studies have revealed a spontaneous re-emergence of PICs that may

significantly contribute to late onset spasticity that develops around post contusion week 6. Accordingly, at post-op week 8, we observed a hyper reflexive pattern in all the velocities tested. These observations indicate that training alone cannot maintain the state of hypo reflexia without any complimentary drug treatment. A similar pattern was seen in the EMG signal at all the different time periods tested and at all the different velocities tested.

Baclofen Control

At post-op week 1, the baclofen control group did not show any hyper reflexive pattern in all the velocities tested. These data suggest that baclofen alone can stabilize the excitatory environment that influences motoneuron excitability immediately after injury, such as cell membrane disruption and an imbalance of inhibitory and excitatory neuromolecules. It is proposed that baclofen maintained an increased concentration of inhibitory neuromolecules by activating the GABA_b receptors and their subsequent contributions to controlling excitation. At post-op week 4, a hypo reflexive pattern was observed due to the above described actions combined with a reduction in availability of unregulated release of excitatory neurotransmitters. However, at post-op week 8, a hyper reflexive pattern was observed in all the velocities tested. This may be due, in part, to the withdrawal effect of baclofen as the baclofen pump was removed at post-op week 4. The withdraw-induced hyper reflexia may be correlated with an increased stretch reflex excitability associated with a significant ligand-mediated down-regulation of GABA_b receptors in the ITB treated spinal cord, a previously reported result of the chronic exposure to baclofen (Kroin et al. 1993). This change also relates to the abrupt loss of the previously described GABA_b associated inhibitory processes. In addition, it may be due to baclofen modulation of the PIC (Heckman et al. 2004), which plays an important role in the development of spasticity; baclofen blocks the Ca²⁺ channels which play an important role in generating the PIC's, and it may have generated PIC's in the absence of baclofen at post-op

week 8. The EMG signals showed the same pattern as that of velocity dependent ankle torque, and it was time locked at all the different time periods tested.

Baclofen Cycle

ITB treatment along with exercise decreased ankle extensor stretch reflex excitability as indicated by significant decreases in velocity dependent ankle torque and time-locked EMG magnitude. During the 1 week period of ITB treatment with bicycle of the lumbar spinal cord, the low-velocity ankle torque was unchanged, whereas torque recorded during high-velocity ankle rotations were significantly decreased compared to pre-treatment values. The reduction in torque was also accompanied by significant reduction in the short-latency EMG that was time-locked to ankle rotation. The reduction in EMG activation is consistent with the increase in the threshold for activation associated with the presumed mechanism of action of GABA_b receptor mediated decrease in transmitter release at the primary afferent terminals (Peshori et al. 1998). A similar finding was reported previously from our lab (Wang et al. 2002). Similarly during post-op week 4 and week 8 the ankle torque and EMG activation were near the pre-treatment values at all the velocities tested.

Current evidence suggests that following the initial trauma, many secondary events including membrane damage, systemic and local vascular effects, altered energy metabolism, oxidative stress, inflammation, electrolyte imbalances, unregulated release of neurotransmitters, and a cascade of biochemical changes affect cellular survival, integrity, and excitability (Tator 1995). From the above data we can see that baclofen, which is a GABA_b agonist, inhibited the release of the excitatory neurotransmitters, which prevented the membrane damage and cellular imbalance in the intracellular and extracellular compartment during the first week post-op, therefore preventing the development of spasticity. During post-op week 4, exercise and baclofen maintained an inhibitory environment and prevented the development of spasticity most likely by

blocking the PIC, which develop in the motoneurons after injury after a brief period of hypo reflexia due to the activation of Na^+ and Ca^{2+} channels, and baclofen prevented it by blocking the Ca^{2+} channels.

At post-op week 8, even after the removal of the drug there was no development of hyper reflexia. This may be due to exercise, which must have maintained the inhibitory environment and stabilized the firing pattern from the motoneurons. It remains to be seen how baclofen works in presence of exercise and how exercise maintains the hypo reflexive state even after removal of the drug. This can be done in future studies by doing single cell motoneuron recordings using patch clamp.

Thus it proves that without baclofen and locomotor exercise treatment spasticity develops immediately after injury and thus proves our hypothesis that acute baclofen and bicycle training is the most effective in preventing the development of the spasticity without deteriorating the condition of the animal as we see from the data that neither alone baclofen or locomotor prevented the development of spasticity at all post-op week 1, 4 and 8.

Open Field Locomotor Assessment

Saline Control

Animals with surgical lesions of the dorsal spinal cord at T₈ that preserved ventral funiculi, demonstrated sufficient self-training that no detectable difference was observed in their locomotor recovery compared with animals that were systematically trained using a treadmill (Fouad et al. 2000). This is true as we also saw a 51% improvement in this group at post-op week 4 when compared to post-op day 1. At post-op week 8 group means BBB scores showed a slight improvement of about 4% from post-op week-4, which is attributed to their intrinsic ability to self-train. Even though the ankle torque data showed spasticity starting from post-op week 1, 4 and 8 this group showed gait improvement without any treatment.

Saline Cycle

This group of animals started with a slightly higher score compared to the other entire group on post-op day 1. It showed an improvement of about 61% at post-op week 4, which is better than the baclofen cycle group. Also, the ankle torque showed hypo reflexive pattern at this time period, which may be due to the exhaustion of the excitatory neurotransmitters as reported earlier or stabilization of the neuronal circuits.

While at post-op week 8, a decrease in BBB score was seen of about 2% from post-op week 4, and also the ankle torque showed hyper reflexive pattern going along with this finding. This may be due to the fact that bicycle training maintained a pattern of uniformity and efficiency, which resulted in the improvement of the BBB score at post-op week 4. Training alone may not be efficient to have a presynaptic inhibitory effect like that of baclofen on the ventral horn motoneurons which may continue to fire continuously leading to hyper reflexia in post-op week 8. After certain period of exercise the improvement in the segmental circuit subserving the muscle and joints may reach the plateau level from where further improvement is not possible. Thus exercise alone may be unable to maintain an inhibitory environment for preventing the development of late onset spasticity and also the gait.

Baclofen Control

The BBB score for baclofen control group improved about 47% from post-op day 1 to post-op week 4, and also it improved about 15% from post-op week 4 to post-op week 8. The score for post-op week 4 corresponds to the ankle torque and EMG data; we observed no spasticity in this group at that period of time. Thus baclofen may have stabilized the cell membrane of the cells after injury and prevented the imbalance in the intracellular and

extracellular environment and thus prevented the development of spasticity and also improved the gait pattern as the BBB score improved.

But, in contrast to ankle torque week 8 data; there was a resurgence of hyperreflexia after the withdrawal of the drug at week 4. The BBB score improved about 14% from post-op week 4. This may be attributable to the preservation of fibers diffusely located in the ventral caudal and ventro-lateral funiculi of the rat spinal cord (Basso et al. 2002; Brustein and Rossignol 1998), or gray matter of the T₁₃-L₂ spinal segments (Magnuson et al. 1999). Even after injury, baclofen may have stabilized them, and after withdrawal of the drug, their connectivity to the muscles and joints of the hind limbs may have improved. Collectively, these may have contributed to the improvement of the BBB score. The ankle torque may be increased due to the withdrawal effect on segmental excitability processes as explained above in the ankle torque data.

Baclofen Cycle

In this group we saw an improvement in the BBB score from post-op day 1 to post-op week 4, a total improvement of 57%. This data corresponds to the ankle torque data where there is no hyper reflexia at post-op week 4, thus it proves that acute locomotor training and baclofen drug prevented the development of spasticity and also improved the gait of the injured animals instead of deteriorating them. In animals and humans with SCI, previous studies have shown improvements in gait parameters following locomotor training using body weight support on the treadmill and manual assistance (Behrman and Harkema 2000; Harkema et al. 1997; Dietz and Harkema 2004) but have not concurrently evaluated effects of bicycle locomotor training following animal with SCI. The findings of the present study are consistent with the suggestions that as therapy, the locomotor training regimen using bicycle, promotes the recovery of walking by optimizing the activity-dependent neuroplasticity of the nervous system (Muir & Steeves

1997; Bose et al. 2005). Neuronal circuits, stimulated by task appropriate activation of peripheral and central afferents via locomotor training, may also reorganize by strengthening existing and previously inactive descending connections and local neural circuits (Muir & Steeves 1997) (Bose et al. 2005).

At post-op week 8, the BBB score improved from post-op week 4, even after removal of the baclofen drug. This proves that the bicycle training may have prolonged the effects of baclofen or it might itself have prevented any secondary damaging effects that may occur after withdrawal of the drug.

Thus, it proves that acute drug therapy and locomotor treatment improves gait and maintains the reflex excitability to the pre-injury values.

Footprints

Hind limb axial rotation and base of support were assessed by footprints, collected while walking along a treadmill, and were measured to compare between cycle trained and control animals. Changes in these parameters in humans have been correlated with dysfunction of descending long tract and propriospinal systems (Kunkel-Bagden et al. 1993).

Saline Control

Saline control group, which received no treatment, did show deterioration in the base of support and axial rotation at post-op week 4, but at post-op week 8, the base of support showed near pre-injury values, while the axial rotation remained similar to post-op week 4 values. As these groups of animals were not bicycle trained and therefore not handled frequently they retained their pre-injury parameters. It has also been shown that control SCI animals have the intrinsic ability to self train.

Saline Cycle

Saline cycle group showed a pattern similar to that of baclofen control with deterioration at post-op week 4 and maintaining the same pattern at post-op week 8. Thus exercise alone may not be beneficial to improve the gait parameters without the drug.

This may lead to one conclusion that whatever changes occur in the segmental circuit that maintain the foot placement and locomotion occur within the first 4 weeks after the injury and it remains the same and does not change later.

Baclofen Control

In this group we saw an increase in base of support and axial rotation at post-op week 4, but at post-op week 8, the values remained more or less similar to post-op week 4.

This may point out that baclofen alone may not be effective in improving the gait parameters and that some form of locomotor training is required as we see an improvement in baclofen cycle at post-op week 4.

Baclofen Cycle

At post-op week 4, the base of support and the axial rotation showed pre-injury values, but at post-op week 8 the base of support and axial rotation showed much increased value compared to the other group. The deficits observed in the baclofen treated animals of the present study could have occurred through changes in the activity of these long tract or propriospinal systems: either at synaptic terminals, cell body of origin, or upon spinal interneurons' modulating posture. It is known that baclofen modulation of the synaptic actions of spinal ventromedial funicular fibers mediated presumably by GABA_b receptors on or near axon terminals and last order spinal interneurons (Jimenez et al. 1991; Quevedo et al. 1992). In addition, GABA_b sensitive sites have been reported in vestibular and functional companion nuclei that regulate the gain of the vestibulospinal reflex (Manzoni et al. 1994). Therefore, it is possible that the ITB treatment

induced changes in posture and limb axis via actions within the spinal cord or within neuronal sites in the brainstem that regulate posture and equilibrium. However, the differences in the time course for the changes in hind limb axis and base of support with improvement in post-op week 4 and deterioration at post-op week 8 may be that after the withdrawal of the drug the GABA_b receptors must have up regulated but there is no ligand (baclofen) present to bind them. But this finding goes against the fact that in baclofen cycle animals the ankle torque showed hypo reflexia at all the time points tested. This could mean that the drug must be acting at different anatomical sites (Wang et al. 2002) to have different effect on ankle torque and footprints. Saline cycle group also did not show much improvement in the footprint parameters at post-op week 8.

One of the possibilities may be that after baclofen pump removal, the toll of exercise may have caused some unwanted stress on the hind limb paws that might have led to external deviation of the paw leading to anatomical defect and thus increase in base of support and axial rotation in post-op week 8. As baclofen pump was removed after post-op week 4, but training was continued up to post-op week 8, the residual effect of baclofen must have prevented the bad effects of exercise. As for the baclofen cycle group, the combination of drug and locomotor therapy prevented the early changes, but once the drug was removed, exercise alone could not maintain the inhibitory environment to prevent the changes from taking place.

Neurotransmitters

Saline Control

Neurotransmitters like 5-HT, GAD₆₇, GABA_b and neurotrophins like BDNF and GAP₄₃ showed a decreased expression when compared to all the other groups. This may be due to the fact that these groups did not receive any locomotor training and baclofen drug. Baclofen, which is a GABA_b receptor agonist, is primarily used as an anti-spastic drug and also the ankle torque and EMG data did not show any decrease in spasticity when their torque amplitude was

measured. Thus these animals showed the least improvement when compared to all the other groups.

Saline Cycle

Neurotransmitters like 5-HT, GAD₆₇, GABA_b receptors and neurotrophins like BDNF and GAP₄₃ did show an increased expression when compared to saline control group. Locomotor training may have facilitated the increased expression of neurotrophins like BDNF and GAP₄₃. As predicted from Cotman's paper (2002) exercise increases the expression of the neurotrophins, which have an effect on the growth of the axons and improves the neuronal plasticity. Since there is little increase in GABA_b receptor expression this may be correlated to the improvement in spasticity seen during post-op week 4. Also, increased expression of 5-HT and GAD₆₇ may correlate with the improvement to the locomotion observed during post-op week 4 on the BBB scale.

Baclofen Control

In this group we also saw similar neurotransmitter expression as that of saline cycle group. As this group received baclofen drug only with no locomotor training, the drug must have influenced the expression of GABA_b receptor till post-op week 4, which also corresponds to the improvement of spasticity during that period. Also, there must be some correlation between 5-HT and GAD₆₇ expression to that of baclofen drug as there was increased expression of the former neurotransmitters. Neurotrophins like BDNF and GAP₄₃ also showed an increased expression to that of saline control group, which co-relates to the improvement in the BBB score observed at post-op week 4. But this group was not able to maintain the improvement till post-op week 8 as the baclofen pump was removed at post-op week 4.

Baclofen Cycle

This group showed the maximum neurotransmitter expression when compared to all the other groups. There was an increased expression of all the neurotransmitters and neurotrophins and also hypo reflexive pattern when tested for spasticity. This may be due to the fact that this group received both baclofen drug and locomotor training. As we have discussed above, exercise improves the expression of neurotrophins like BDNF and GAP₄₃, which promotes neuronal plasticity and improved neuronal growth in case of injury, thus leading to improvement in gait and spasticity. Baclofen drug increased the pre-synaptic inhibition by increasing the expression of GABA_b receptors, which was responsible for the hypo reflexive response seen during measurement of spasticity.

Thus, all the above data show that if baclofen drug and locomotor training in the form of bicycle, if started acutely after spinal cord injury, may prevent the development of spasticity and help in improving the gait of the animal. By contrast, baclofen alone or cycle alone did not show a continuous maintenance of hypo reflexive pattern, with later development of spasticity and not much improvement in the gait.

CHAPTER 6 CONCLUSION

In humans, one of the most devastating chronic effects following SCIs is the development of spasticity. Spasticity leads to velocity dependent lengthening resistance in the muscles resulting in contractures and secondary biochemical changes which eventually cause destruction of the muscles. In humans the present antispastic treatment is the continuous infusion of ITB, a GABA_B agonist that acts on the presynaptic receptors leading to inhibition of both monosynaptic and polysynaptic reflexes. At present, there are no approved guidelines to administer this drug acutely before the symptoms of spasticity become evident. Locomotor training in the form of weight bearing stepping using treadmill has been shown to improve the gait of the SCI patients. Treadmill training requires extensive use of manpower to hold the patient while training and also this facility is present only in few selected locations across the nation. Therefore, the aim of this thesis was to provide preclinical data to test the efficacy of baclofen therapy with or without locomotor training (bicycle locomotor training) which has already been shown to be as effective as treadmill (Bose et al. 2004).

To test the above hypothesis spinal cord injured rats were used as their spinal cord circuitry resembles to that of human. Moreover, contusion injury mimics most common SCI in humans and produces spasticity. The animals were divided into 4 groups after contusion spinal cord injury:

Saline control group - saline pump, no bicycle training

Saline cycle group - saline pump, bicycle training

Baclofen control group - baclofen pump, but no bicycle training

Baclofen cycle group - baclofen pump + bicycle training.

The animals were selected only if their open field locomotor (BBB) score fell below 3 when tested at post-op day 1. The animals were trained over the course of 2 months. The training schedule was performed 5 days a week using two 20 minute trials/day, starting from post-op day 1. On the first day of training, the rats were given five minutes training.

The baclofen and saline pump were removed at post-op week 4. The animals were tested for spasticity at post-op weeks 1, 4 and 8 using the velocity dependent ankle torque set up developed in our lab (Thompson et al. 1996; Bose et al. 2002) and also measuring the EMG signals from the muscles in the same set-up. The animals were tested for gait improvement using the BBB scale and footprints at post-op weeks 4 and 8. The animals were then sacrificed using 4% freshly prepared paraformaldehyde using an accepted protocol to remove the thoracic, injured and lumbar spinal cord. The spinal cords were then used to do immunofluorescent experiments to test the presence of various neuromolecules such as GABA_b, GAD₆₇, 5-HT, BDNF and GAP₄₃ which have documented roles in the motoneuron excitability and neuroplasticity following injury and therapy.

As per the results we got from all the above experimental procedures we can come to the conclusion that acute baclofen drug therapy with bicycle training prevented the development of spasticity measured by velocity dependent ankle torque and associated EMG data. Moreover, this combined treatment showed an increment in BBB score compared to post-op day 1 when compared to other 3 experimental groups. The immunofluorescent images showed qualitative increase of GABA_b receptors as well as other neuromolecules such as 5-HT, GAD₆₇, BDNF and GAP₄₃ as compared to all the other 3 groups. Hypo reflexive pattern seen in baclofen cycle group might be related to GABA_b receptors mediated presynaptic inhibition of the stretch reflexes. However, interestingly, the base of support and axial rotation did not show improvement when

compared to other groups. While the control groups such as baclofen drug alone or saline cycle alone did show some decrease in spasticity at post-op week 4, they could not retain the same improvement at post-op week 8. Thus to conclude, we found that acute baclofen drug therapy along with bicycle training prevented the development of spasticity. Moreover, the acute training and drug therapy did not deteriorate the condition of the injured animals. Therefore, these preclinical data have the potential to translate in human clinical trial.

CHAPTER 7 FUTURE WORK

In this research project, we have shown that combination therapy of baclofen drug and locomotor training started acutely after spinal cord injury prevented the development of spasticity and improved the gait, and overall locomotor capacity. Surprisingly, the individual therapy of either drug (ITB) or locomotor training (customized bicycle) did not show benefit in improving these condition. Baclofen and locomotor exercise possibly work synergistically and thus, the mechanism underlies these benefits need to be unfolded. Therefore, a detailed study involving GABA_b receptor profile as well as properties of the motoneuron has to be investigated by intracellular motoneuron recording using *in vivo* patch clamp during different time periods of the treatment. As this research only conducted qualitative analysis of the GABA_b receptors by immunofluorescent technique, molecular techniques such as Western Blot or ELISA can be used to quantitatively measure the expression of receptors in the spinal cord tissue to test differences in expression in different groups. Moreover, neurotropic factor, such as BDNF, is an important indicator of regeneration of injured axons which mediates neuroplasticity. Therefore, a detailed molecular study to investigate the profile of BDNF and its receptor trkB can provide a better understanding of neuroplasticity mediated by BDNF following locomotor training. Furthermore, *in vivo* longitudinal MRI study using volumetric measurement of the lesion can be done to further predict the benefits of this combined therapy. At present, I am planning to work on post fixed spinal cord tissue using T₂-weighed MRI imaging to study the effects of locomotor and drug therapies. These multi-dimensional studies may further enhance our understanding in the mechanism of the recovery/benefits we have observed following locomotor and drug therapies.

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BIOGRAPHICAL SKETCH

Rita Jain was born in Mumbai, India. She completed her bachelor's degree in medicine in 2001. After her graduation she worked as a resident medical officer in Niramaya Hospital in Pune, India. After working there for 6 months, she realized that her knowledge was not sufficient to bring a breakthrough in the medicine world and to decrease the suffering of the patients. She decided to go for higher education. She came to United States in 2003, to pursue her master's degree in medical sciences at the University Of Florida. She chose to specialize in neuroscience. For the next 3 years, Thompson's lab was her home. There she worked under the guidance of Dr. Prodip Bose and Dr. Floyd Thompson on various research projects.

