MAGNETIC RESONANCE CHARACTERIZATION OF SKELETAL MUSCLE ADAPTATIONS AFTER INCOMPLETE SPINAL CORD INJURY

By

PRITHVI KRISHNAKANT SHAH

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To My Uncle, Dinesh O Shah

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I can no other answer make, but, thanks, and thanks.
~William Shakespeare

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LIST OF ABBREVIATIONS

ASIA	American spinal cord injury association
[ADP]	Absolute concentration of free cystolic adenosine diphosphate
[ATP]	Absolute concentration of adenosine triphosphate
[ADP][Pi]/[ATP]	Phosphorylation potential
CSA	Maximum cross sectional area
EMCL	Extramyocellular lipid
EMS	Electrical muscle stimulation
End ex	End exercise (EMS)
FID	Free induction decay
GAS	Gastrocnemius muscle
^{1}H-MRS	Proton magnetic resonance spectroscopy
IMCL	Intramyocellular lipid
k_{PCr}	Rate constant of PCr recovery
LT	Locomotor training
mM	milimoles/liter of intracellular water
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
[Pi]	Absolute concentration of inorganic phosphate
[PCr]	Absolute concentration of phosphocreatine
^{31}P-MRS	Phosphorus magnetic resonance spectroscopy
Q_{max}	Maximum oxidative ATP synthesis rate
$Q_{max\text{-}ADP}$	Maximum oxidative ATP synthesis rates based on k_{PCr} and [ADP]

$Q_{max\text{-}[ADP][Pi]/[ATP]}$	Maximum oxidative ATP synthesis rates based on k_{PCr} and phosphorylation potential.
SCI	Spinal cord injury
T2	T2 relaxation times
TR	Repetition time
V_{ex}	Extrapolated initial rates of PCr recovery (k_{PCr}*ΔPCr)
V_{dep}	Rates of PCr depletion at onset of EMS
$V_{max\text{-}lin}$	Maximal rate of PCr resynthesis
V_{meas}	Initial rates of PCr recovery (first three points in recovery)
ΔPCr	$[PCr]_{rest} - [PCr]_{end\ ex}$
(μmol/g wet wt)	micromol/gram wet weight

Abstract of Dissertation Presented to the Graduate School
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By

Prithvi Krishnakant Shah

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Spinal cord injury is one of the most disabling health related problems that often results in paralysis and paresis of body musculature below the lesion site. Persons with incomplete-SCI typically exhibit impaired motor performance and varying degrees of functional limitations. Despite the obvious motor dysfunctions, physiological muscle adaptations following incomplete-SCI are relatively unstudied. An understanding of the muscular adaptations following an incomplete-SCI will help in the development of therapies aimed at reducing the secondary effects of paralysis and paresis. The overall objective of this dissertation was to investigate skeletal muscle adaptations following incomplete-SCI using combinations of non-invasive MRI and MRS techniques.

Findings from our human studies reveal that chronic incomplete-SCI is associated with significant muscle atrophy in the affected lower extremity that is uniform between limbs and somewhat influenced by mobility status. In addition, persons with incomplete-SCI demonstrate an increase in the total lipid, IMCL and extramyocellular EMCL content and enhancements in the T_2 relaxation properties of the lower leg muscles. Moreover, repetitive locomotor training with body weight support and a treadmill are associated with significant increases in the

plantarflexor muscle size. Data from our animal experiments reveal that the paralyzed rat hindlimb muscle show faster rates of PCr depletion, thereby suggesting that, after SCI, there is either an increase in ATP requirement for similar demands in muscle contraction or the overall supply of ATP is compromised following the injury. In addition, a pronounced decrease in PCr recovery rates implies a less effective oxidative phosphorlyation and a reduction in the mitochondrial oxidative capacity of skeletal muscle. Collectively, findings from this dissertation work reveal that the paralyzed skeletal muscle shows drastic alterations in its morphological and metabolic properties after a SCI and that these adaptations can be successfully characterized by the use of non-invasive MR techniques.

The present work will provide a foundation from which the relationship between skeletal muscle adaptations and function in this population can be further explored. Moreover, the use of sophisticated MR techniques will enable characterizing the paralyzed muscle non-invasively and with high resolution; while also allowing longitudinal follow-ups - all of which are crucial in the assessment of injury mechanisms, disease progression and efficacy of therapeutic interventions.

CHAPTER 1
SPINAL CORD INJURY AND SKELETAL MUSCLE

1.1 Significance

Spinal cord injury (SCI) results in paralysis and paresis of muscles below the injury site making movement difficult. Impairments in skeletal muscle contribute to a host of musculoskeletal deficits that lead to secondary health related conditions that cost an estimated $220,000 and $750,000 per person annually (NSCISC 2006). The clinically relevant musculoskeletal and movement disorders in persons with SCI include muscle weakness and paralysis of affected extremities (Gordon and Mao 1994; Kim, Eng et al. 2004), early muscle fatigue and increased energy demands for simple functional activities (Hopman, Dueck et al. 1998; Ulkar, Yavuzer et al. 2003) , diminished capacity or inability to ambulate (Burns, Golding et al. 1997; Stein, Chong et al. 2002), and overall decreased endurance and dependence on assistive aids for locomotion (Waters, Adkins et al. 1994; Ulkar, Yavuzer et al. 2003). Furthermore, inactivation of paralyzed skeletal muscle interferes with implementation of therapeutic interventions (Subbarao 1991). Inactivity and sedentary lifestyle in this patient cohort leads to unloading of paralyzed muscles. As a result, a myriad of muscle adaptations including morphological, contractile, metabolic and neural alterations ensue following SCI (Castro, Apple et al. 1999; Shields 2002). Note worthily, most of these adaptations have been studied following a complete SCI. Understanding these muscular adaptations following an incomplete SCI will help in the development of therapies aimed at reducing the secondary effects of paralysis and paresis. The purpose of this work is to investigate skeletal muscle adaptations following incomplete SCI using non-invasive magnetic resonance imaging (MRI) and spectroscopy (MRS) measures. Combinations of human and animal models of incomplete SCI are utilized to achieve this goal.

1.2 Spinal Cord Injury in Humans

The current gold standard classification system for SCI, *American Spinal Injury Association Classification System - ASIA*, is based on the presence, absence and degree of normality of motor and sensory impairments (ASIA 2001). Since these impairments are highly variable, classifying SCI has posed a major challenge for clinicians. According to the *ASIA* classification system, human SCI is classified as either complete or incomplete. Complete SCI involves "total absence of sensory and motor functions in the lowest sacral segments", whereas an incomplete SCI is characterized by partially preserved motor and/or sensory function below the level of lesion, including sparing of the lowest sacral segments (ASIA 2001). Irrespective of the severity of injury, the majority of human spinal cord injuries leave the spinal cord tissue relatively intact (Kakulas 1987). As a result, often after infarction, contusion and/or mechanical deformation, much of the spinal cord tissue is spared. A severed cord is seen only in rare injury types such as sword injuries, bullet wounds or penetration of bone fragments into the cord. Interestingly, while some individuals with significant sparing of spinal cord tissue may present as "clinically complete," others with very little spinal cord tissue sparing are considered "incomplete". These varying clinical signs make classifying SCI difficult and leaves clinicians "guessing" as to the integrity of the spinal cord tissue.

With technological advancements in the management of acute SCI, and with an increased life expectancy of persons with an incomplete injury, there is an emerging healthcare trend in the treatment of persons with chronic SCI (NSCISC 2006). The majority of new spinal cord injuries (~53%) occurring annually are now classified as incomplete. Given their unique injuries and varying degrees of tissue sparing, persons with incomplete SCI constitute an extremely heterogeneous group. Individuals after this type of injury exhibit a continuum of ambulatory abilities ranging from being completely wheelchair dependent to nearly normal walking without

the use of assistive devices (Waters, Adkins et al. 1994; Melis, Torres-Moreno et al. 1999). Accordingly, due to their variable paralysis and paresis, they present with impaired motor performance and varying degrees of functional limitations (Subbarao 1991; Tang, Tuel et al. 1994; Burns, Golding et al. 1997). Despite the obvious motor dysfunctions in persons with incomplete SCI, muscle adaptations following the injury have not been well described.

1.3 Spinal Cord Injury in Animal Models

The benefits of utilizing experimental animals is reflected in the more than hundred years of ongoing research in animal models of SCI. Current understanding of anatomical and molecular changes in response to human SCI has largely been derived from parallel lesion models of experimental SCI in animals that mimic human SCI, both histologically and behaviorally. Animal models of SCI enable investigation of SCI at a level of detail that would not be possible in human studies. A variety of experimental animal models of adult SCI involving rats, mice, pigs, monkeys and cats have been utilized for this purpose (Wrathall 1992; Anderson, Howland et al. 1995; Qayumi, Janusz et al. 1997; Stokes and Jakeman 2002; Rosenzweig and McDonald 2004; Ni, Li et al. 2005). The majority of these studies have utilized various injury paradigms ranging from sharp transections to blunt contusion of the spinal cord that simulate the variations in the severity of human SCIs. Animal models of SCI have provided insights into, and continue to contribute greatly towards our current understanding of the human spinal cord injury mechanisms, anatomical and pathophysiological sequels of the injury, neuromuscular adaptations following the injury, regeneration and repair of spinal cord, and effectiveness of novel therapeutic interventions following injury. Furthermore, investigations have also attempted to answer the question of effective duration and time frames of implementing these interventions (Rosenzweig and McDonald 2004). Goals and objectives of a study dictate which animal and lesion model is best suited for a given study.

1.3.1 Spinal Cord Injury in the Rat Model

Of the various animal models mentioned above, research on studying the rat model of SCI has gained tremendous momentum in the recent years (Wrathall 1992; Kwon, Oxland et al. 2002; Young 2002). In the 1980s, the feline spinal cord model dominated the field of SCI research in investigating the wide gamut of neurophysiological changes including biochemical, physiological, vascular and metabolic changes following the injury (Young 2002). In fact, the cat spinal model yielded several therapies that went to clinical trial in humans. One of the most classic examples is the first clinical trial of methylprednisolone in 1985 (Bracken, Shepard et al. 1985). However, in recent years the trend has been towards the use of rats for SCI studies. Two major interconnected determinants of this transition have been recognized. Firstly, greater percentages of SCI in humans are now being classified as incomplete injuries. Consequently, the weight drop device similar to that used for the feline spinal cord contusion model was developed by Noble and Wrathall in 1987 for use in rats. This device induces spinal cord contusion in rats that morphologically and behaviorally mimics human incomplete spinal cord injuries (Wrathall, Pettegrew et al. 1985; Noble and Wrathall 1989) (see below for more details). Secondly, rat models are advantageous because they are easily accessible and large groups of rats are easy to manage in a laboratory setting. Rat models allow for practical post-operative animal care, conduction of longitudinal studies in a feasible time frame due to relatively shorter lifespan of animals, and considerably lower initial costs (Khan, Havey et al. 1999); while simultaneously serving the purpose of mimicking an injury that corresponds to human SCI. Assessment of post-injury symptoms in the rat model is reliably and validly tested using the Basso Bresnahan, Beattie scale (BBB) (Basso, Beattie et al. 1995).

Lastly, with the explosion of genetic research in neuroscience, SCI mice models are becoming more prevalent (Stokes and Jakeman 2002). However, this model has not as well been

validated and suffers from relatively larger variability in injury production and locomotor functional assessments post-injury (Rosenzweig and McDonald 2004; IOM 2005).

1.3.1.1 Spinal cord contusion model in rats

The common experimental rat models of SCI include transection, compression and contusion (Kwon, Oxland et al. 2002; Rosenzweig and McDonald 2004). Each of these models exhibit unique characteristics and are used as per the experimental objectives. A transection rat model produces complete injury to the spinal cord and is more commonly used to study the neurophysiological processes/mechanisms above or below cord lesion sites. Transection model studies overcome the confounding effects of spared neural pathways that are commonly present in studies using incomplete injury models. A compression model, on the other hand, causes cord compression and better simulates ischemic injuries to the spinal cord. This model is commonly used to study the acute vascular pathophysiology following SCI (Kwon, Oxland et al. 2002; Young 2002). Finally, the SCI contusion model produces mechanical impaction of the cord and most closely imitates an incomplete SCI in humans both pathophysiologically and behaviorally and can create graded focal injuries similar to humans (Metz, Curt et al. 2000). In the present dissertation work, SCI contusion injury in rats is utilized as the model of choice.

Various approaches have been devised to induce an experimental contusion injury in the rat model including weight drop impaction of an exposed spinal cord (Wrathall, Pettegrew et al. 1985), contusion by compressing the cord between the arms of an aneurysmal clip (Rivlin and Tator 1978), arterial occlusion ((IOM 2005) or chemical toxicity (Magnuson, Trinder et al. 1999). Of these, induction of contusion injury to the cord by impaction has proven to be most relevant to incomplete, traumatic SCI in humans. The mechanical impact by a weight drop on the exposed cord reflects the dynamics of clinical injury that occurs in humans (Khan, Havey et al. 1999; Metz, Curt et al. 2000; Kwon, Oxland et al. 2002; Young 2002). The majority of human

incomplete spinal injuries are a direct result of trauma to the cord. Traumatic SCI in humans causes an initial mechanical insult to the cord that involves a primary lesion, termed the umbra region, followed by a penumbra or secondary injury that involves either further damage to already damaged tissue or new damage to otherwise healthy tissue (Hall and Springer 2004). The underlying molecular and pathophysiological mechanisms and the functional symptoms following an incomplete SCI in humans are well reproducible in the *contusion* rat model. In fact, electrophysiological outcomes such as motor evoked potentials (MEP) and somatosensory evoked potentials (SSEP), spinal cord lesion morphology, relationship between the morphology and functional measures such as functional locomotor capacity have been demonstrated to be analogous in humans with SCI and spinally contused rats (Noble and Wrathall 1985; Noble and Wrathall 1989; Metz, Curt et al. 2000). As a result, the contusion animal model of SCI has been established as a valid model of SCI for comparison between rats and humans.

1.3.1.2 Spinal contusion by impaction

The weight drop device used to induce spinal contusion injury was first introduced by Allen in 1911 where weight was dropped onto canine cords exposed by laminectomy (Young 2002). Unfortunately, Allen's death in World War II created a void in the use of this model. It was only in the late 1960s, that investigators revived the contusion model in the primate and feline spinal cord to describe the histopathological effects, evoked potentials and assess effects of corticosteroids and hypothermia in response to SCI. However, owing to questionable reproducibility of the weight drop technique, a standard graded and reproducible spinal contusion injury procedure by impaction was warranted. By making definite alterations in the height at which a 10g weight was dropped on the exposed dura, a reproducible injury to the rat spinal cord was established by Wrathall et al in 1985 (Wrathall, Pettegrew et al. 1985). Depending upon the desired severity of injury to the cord, the 10g weight is dropped from

heights of 2.5cm, 5cm or 17.5 cm on the exposed dura. Accordingly, injury to the cord is considered mild, moderate or severe respectively, corresponding to similar severity of spinal injuries in humans.

Before experimental therapeutic interventions can be implemented, standard protocols are required to help minimize variability in injury between animals. Indeed, the magnitude of mechanical injury produced by these methods have been validated and proven to closely correlate with histological, behavioral, electrophysiological evaluations and functional measurements following SCI (Gale, Kerasidis et al. 1985; Noble and Wrathall 1985; Metz, Curt et al. 2000). Three standard devices are now used to produce this injury in rodents: Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor formerly called the New York impactor; the Ohio State University impactor and the Infinite Horizons device (Wrathall 1992; Basso, Beattie et al. 1996; Khan, Havey et al. 1999; IOM 2005).

1.3.2 Assessment of Locomotor Behavior in Rats

One of the first behavioral test scores to assess recovery in spinal injured animals was established by Tarlov and Klinger in 1954. Briefly, the testing used a six-point scale to assess motor function recovery after spinal injury in dogs (grade 0 = no spontaneous movement; grade 5 = normal motor function) (Tarlov and Klinger 1954). The Tarlov score, as it is commonly named today, has emerged as a popular tool to assess motor recovery in a variety of spinal injured animals including rats. In addition, various other motor scoring approaches have been specifically formulated for the spinal injured rat. Bresnahan et al in 1987 compiled a behavioral testing strategy that included assessments of general locomotor skill (in an open field), fine locomotor skills (through grid walking task) and postural adjustment to displacement (in an inclined plane) after SCI in rats (Bresnahan, Beattie et al. 1987). However, a valid and reliable

tool – the Basso, Beattie and Bresnahan score, also commonly known as the BBB score was launched only in 1995 (Basso, Beattie et al. 1995).

The BBB score is an operationally defined ordinal scale that assesses hind limb locomotor recovery of thoracic spinal cord injured rats. It is the first assessment tool that has been validated with testers from several laboratories across the United States (Basso, Beattie et al. 1996). Each of the components of the 21 point scale is based on specific features of locomotor recovery after spinal cord contusion in rats including joint movements, trunk posture, weight support, stepping and weight support ability, coordination between forelimbs and hind limbs, and tail position. The score order from 0 to 20 assumes progressive recovery with every score representing a unique and sequential stage of locomotor recovery: a) scores 1 through 8 center around recovery of joint movements unique to the early phase of recovery, b) scores 9-13 focus upon initial recovery of stepping ability and coordination that represents an intermediate stage of recovery and c) scores 14-21 focus on the progression in foot placement, toe clearance trunk stability and tail position during stepping that represents the late and final phase of recovery. Testing consists of placing the animal in an open field beginning as early as one day post injury and observing it for 4 minutes. Behavioral scoring is done by two observers in real time with repeated testing every week for 6-9 weeks. Used extensively throughout the neurotrauma literature, the scoring system is sensitive enough to differentiate between severities of the spinal injury including mild to severe spinal contusion and transection (Basso, Beattie et al. 1996).

Though the versatility of the BBB score makes it a valuable locomotor assessment tool, investigators have also questioned its accuracy in assessing specific categories such as coordination. Accordingly, measures such as the Catwalk analysis have been proposed as potential addendums to the standard BBB scale (Koopmans, Deumens et al. 2005). The Catwalk

analysis allows collection of data on dynamics of locomotion such as *degree* of coordination and weight bearing, duration of gait cycles etc. In addition, various other measures have been identified to assess locomotor recovery after SCI in the rat; the use of which depends on the research question posed by investigators. The inclined plane test for example measures the animals' ability to maintain position in an inclined plane and is commonly used to assess balance and posture (Rivlin and Tator 1977; Bresnahan, Beattie et al. 1987). The crossway tests involves crossing runways such as a beam or grid (Kunkel-Bagden and Bregman 1990). The substantial motor control required to pass this test makes it suitable for assessing limb coordination. Video recordings from these tests yield data such as time taken and number of errors made during crossing. Treadmill walking is yet another measure of assessing locomotor recovery and is often used for assessment of footprint and kinematical analysis of individual joint movements (Kunkel-Bagden and Bregman 1990).

1.4 Skeletal Muscle Adaptations after SCI

Skeletal muscle adaptations following SCI are a direct consequence of neuronal damage within the spinal cord and an indirect result of prolonged periods of muscle inactivation. In addition, paralyzed skeletal muscle is subject to altered loading conditions and muscle length (Alaimo, Smith et al. 1984; Gordon and Mao 1994; Dietz, Colombo et al. 1995; Shields 2002). Ultimately, a myriad of muscle adaptations including morphological, contractile and metabolic muscle alterations occur following SCI. The following discussion is focused on adaptations of paralyzed muscles that are partially or completely devoid of a descending spinal drive and yet have an intact peripheral nerve supply.

1.4.1 Morphological Adaptations: Muscle Atrophy

Like any other model of muscle disuse, such as cast immobilization (Vandenborne, Elliott et al. 1998; Stevens, Walter et al. 2004), unilateral lower limb suspension (Berg, Dudley et al.

1991; Hather, Adams et al. 1992), bed rest (Berg, Larsson et al. 1997; Alkner and Tesch 2004), space flight (Akima, Kawakami et al. 2000; Tesch, Berg et al. 2005) and inactivity secondary to injury or disease processes (Arokoski, Arokoski et al. 2002; Johansen, Shubert et al. 2003), SCI is accompanied by marked atrophy of the paralyzed muscles. The abovementioned human models of disuse atrophy result in 15% - 32% decrease in muscle size of the immobilized lower extremity muscles, depending upon the muscles tested and time of testing. A number of studies have reported effects of disuse ranging from immobilization periods of 1 week to almost 17 weeks. Generally, greater declines in muscle size are reported for postural and antigravity muscles such as the soleus and vasti versus the knee and ankle flexors of the lower extremity (Hather, Adams et al. 1992; Akima, Kawakami et al. 2000; Tesch, Berg et al. 2005). In comparison to other disuse models, atrophy of the paralyzed muscles following SCI is markedly higher. An overall 40-80% decline in the average CSA of human lower extremity muscles has been reported 24 weeks after complete SCI. The degree of atrophy typically depends upon the functional role, shortened muscle length related to posture assumed by paralyzed limbs and anatomical location of muscles (Castro, Apple et al. 1999). Using magnetic resonance imaging (MRI) techniques, Castro et al reported that the antigravity plantarflexor muscles show greater decline in CSA as compared to the dorsiflexors (40% versus 20%). On the other hand, the thigh antigravity knee extensors and knee flexors, unlike in other models of muscle disuse (Tesch, Berg et al. 2005), show similar degrees of atrophy (~42%) (Castro, Apple et al. 1999). While this initial study by Castro et al reported the muscle CSA inclusive of fat within the muscle, the same research group subsequently reported an almost 38% decline in total thigh fat-free muscle CSA of the injured group as compared to controls (Elder, Apple et al. 2004). Modelesky et al also estimated a 38-44% decline in the fat-free muscle mass in individuals with complete SCI

two years after injury using MRI and Dual X-Ray absorptiometry (Modlesky, Bickel et al. 2004). Other investigators have studied alterations in muscle fiber size based on needle biopsies. Declines of 54-74% in muscle fiber size are observed by 24 weeks after SCI with maximum declines seen in muscle fiber types that have a greater fiber CSA initially (i.e. fast twitch fibers) (Scelsi, Marchetti et al. 1982; Castro, Apple et al. 1999). While maximum rates of decline in whole muscle size (60%) and muscle fiber size (74%) are seen during the first 6 weeks after injury, progressive atrophy is observed for as long as one year after SCI (Scelsi, Marchetti et al. 1982). Furthermore, atrophy appears to be muscle specific: the gastrocnemius muscle, for example, continues to atrophy for as long as 6 months, whereas the tibialis anterior and semimembranosus reach a plateau by as early as 6 weeks after injury (Castro, Apple et al. 1999). While maximum emphasis has been placed on studying atrophic adaptations in the lower extremity paralyzed muscles, a couple of studies have also reported atrophy of upper extremity muscles (Thomas, Zaidner et al. 1997) and abdominal muscles (Estenne, Pinet et al. 2000) following SCI.

Atrophy following animal models of SCI is in concurrence with humans with SCI where almost 10% to 56% of whole muscle and fiber atrophy is observed depending upon the muscles studied and the model of injury based on injury severity (Roy and Acosta 1986; West, Roy et al. 1986; Dupont-Versteegden, Houle et al. 1998; Gregory, Vandenborne et al. 2003; Otis, Roy et al. 2004). Similar to human studies, larger degrees of atrophy are observed in the slow antigravity soleus muscle as compared to the fast flexor muscles such as the tibialis anterior or extensor digitorum longus. Moreover, similar to humans, antigravity muscles continue to atrophy for at least 6 months after SCI. However, unlike the paralyzed knee flexors in humans, minimal declines are seen in the non-antigravity knee flexor muscles (semitendinosus) following SCI in

animal models (Roy and Acosta 1986; Roy, Talmadge et al. 1998; Otis, Roy et al. 2004). **While all the above-mentioned studies have focused on the complete SCI human and animal models, no studies in humans and just few spinal contusion animal model investigations have reported the impact on skeletal muscle after incomplete spinal cord injuries.** Hutchinson et al studied lower extremity muscle atrophy following contusion injury in rats over the course of ten weeks (Hutchinson, Linderman et al. 2001). At one week, they found a 20-25% decline in the wet weight of lower leg muscles (soleus, plantaris, gastrocnemius and tibialis anterior) and no change in the EDL muscle wet weight. While spontaneous recovery occurred in the soleus, plantaris and tibialis anterior muscles by three weeks, the gastrocnemius muscles continued to show declines for as long as ten weeks (Hutchinson, Linderman et al. 2001). Similarly, Liu et al have reported an 11%-26% decline in the triceps surae and tibialis anterior cross sectional areas two weeks after spinal contusion injury. Spontaneous recovery in the paralyzed muscle was seen by four weeks (Liu, Bose et al. 2008).

In the present work, muscle cross-sectional area of paralyzed lower extremity muscle is studied in humans with incomplete SCI. In addition, the impact of ambulatory status of persons with incomplete SCI on muscle size is determined. A detailed explanation of this study follows in Chapter 6.

1.4.2 Morphological Adaptations: Muscle Fiber Type Conversion

Muscle fiber type conversion is another hallmark of chronically paralyzed skeletal muscle. Muscle fibers can be categorized using several classification systems. The most common and recent refinement for characterization of muscle fibers is based upon the myosin heavy chain (MHC) expression of muscle fibers. MHC is a critical structural and enzymatic muscle protein that possesses distinct molecular forms (isoforms). MHC isoforms control the pH of the myosin ATPase reaction and accordingly are responsible for the degree of histochemical staining of

muscle fibers during immunocytochemistry (McComas 1996). Depending upon the staining, four main isoforms of the MHC corresponding to I, IIa, IIx, IIb fiber types have been identified in the mammalian skeletal muscle. Thus, fibers that stain strongly at pH 9.4 are referred to as type II in contrast to the poorly reacting type I fibers (McComas 1996). Type II fibers are further classified as IIa, IIb or IIx on the basis of the strength of staining. (Note: IIb fiber type in humans is now termed as type IIx fibers rendering humans without type IIb fibers). Importantly, MHC isoforms also determine the rate of cross-bridge reactions with actin filaments and therefore the speed of muscle shortening. Accordingly, type I fibers generally are the slow twitch fibers that generate twitch forces slowly and are less fatigable. By and large, type I fibers possess relatively higher concentrations of oxidative phosphorylation enzymes, mitochondrial content, capillary density and redox proteins; thereby making them more suitable for sustained periods of ATP production. Type IIb fibers on the other hand quickly generate peak tension and are easily fatigable. They are generally equipped with relatively larger concentrations of glycolytic enzymes, phosphocreatine, glycogen, calcium sequestrating proteins and sarcoplasmic reticulum; thereby making them more suitable for burst activities that demand rapid production of ATP. Fast twitch fibers contain relatively lower mitochondrial content, blood capillaries and hence redox proteins. Finally, Type IIa and IIx muscle fibers possess intermediate characteristics. Many studies also report existence of a mixed/hybrid phenotype such as type 1/IIa and IIx/IIb; especially in paralyzed muscles (Roy, Talmadge et al. 1999). While the above-mentioned classification is confined to muscle fibers, categorization of whole muscle is not uncommon. Whole muscles are generally termed as slow or fast twitch based upon the proportion of fiber types, though both fiber types co-exist in the same muscle. Thus, a slow muscle is generally recognized as having relatively greater oxidative capacity, predominantly consists of type 1 fibers, is functionally more fatigue resistant,

has slow twitch muscle properties (see below) and participates in slow phasic muscle activities. Based on the resting content of bioenergetically important metabolites, slow muscles also have relatively higher Pi/PCr ratios as compared to white muscles; the differences being largely due to a higher Pi content (Meyer, Brown et al. 1985; Kushmerick, Moerland et al. 1992). Examples of such a muscle include the soleus and vastus medialis. A fast muscle in contrast, demonstrates an overall faster contractile property, is more fatigable, with relatively lower mitochondrial oxidative capacity and larger glycolytic capacity, predominantly consists of type II fibers and plays a major role in fast tonic activities (such as sprinting). Metabolically, fast muscles have relatively lower Pi/PCr ratios as compared to slow muscles; the differences being largely due to a higher PCr content (Kushmerick, Moerland et al. 1992). Examples include the gastrocnemius and vastus lateralis muscles. While skeletal muscles of animals are more homogenous in the fiber type composition and characteristics, the human skeletal muscle is extremely heterogeneous.

Usually, muscle inactivity due to disuse, immobilization or even decreased neural activity causes muscle fiber type conversion from the slow to fast phenotype. Reverse expression of muscle fiber type is observed with increased muscle activity. There is a decrease in the expression of type I muscle fibers and an increase in the proportion of hybrid type I+IIa and pure type IIb and/or IIx muscle fibers after chronic SCI in both animal models (Mayer, Burke et al. 1984; Lieber, Friden et al. 1986; West, Roy et al. 1986; Roy, Talmadge et al. 1999; Otis, Roy et al. 2004) and in humans (Scelsi, Marchetti et al. 1982; Lotta, Scelsi et al. 1991; Burnham, Martin et al. 1997; Castro, Apple et al. 1999). Similar to complete SCI, recent studies using the rat model of incomplete SCI have also demonstrated a similar shift in fiber type expression with declines in the levels of type IIa, elevations in type IIb MHC along with the presence of a

transitional type IIx MHC (that is normally not seen in the control soleus muscle) after incomplete injuries (Hutchinson, Linderman et al. 2001; Stevens, Liu et al. 2006). In incomplete models of SCI, this conversion occurs as early as 1-3weeks and is reversible (Hutchinson, Linderman et al. 2001). This fiber type conversion after SCI leads to significant alterations in the characteristics of paralyzed muscle including a change in contractile properties, metabolic capacities and an impaired endurance capacity (Hopman, Nommensen et al. 1994; Burnham, Martin et al. 1997).

1.4.3 Adaptations to Contractile Properties

After SCI, muscles with a greater proportion of type I fibers and slower contractile properties (slow twitch muscles) generally exhibit characteristics analogous to those of muscles with a greater proportion of type II fibers and relatively faster contractile properties (fast twitch muscle). Most human and animal studies concur that the contraction and relaxation speeds of slow twitch muscle, but not fast twitch muscles, are significantly faster following chronic SCI (one year after injury) when compared to before injury (Lieber, Friden et al. 1986; Roy and Acosta 1986; Gerrits, De Haan et al. 1999; Roy, Talmadge et al. 1999; Shields 2002).

In the spinalized animal model, contractile properties of the paralyzed soleus muscle (an otherwise slow muscle) are consistent with those of a primarily fast muscle. Elevations in the slow muscle contraction speeds after SCI are reflected as decreases in time to peak tension (by almost 20ms), increase in specific tension (by almost 100%), and increases in fusion frequency (by almost 100%). In addition, paralyzed muscle demonstrates faster twitch half-relaxation times (Lieber, Johansson et al. 1986; Roy and Acosta 1986). While adaptations following a complete SCI are quite drastic, contractile properties after an incomplete SCI are relatively less marked. A decrease in half- relaxation time by 20% in the soleus muscle that recovers to control values by ten weeks is reported in rodents with a moderate spinal cord contusion (Stevens, Liu et al. 2006).

Though conversion of muscle fiber type is generally purported as reasons for alterations in contractile properties, various molecular mechanisms are suggested for this change. These mechanisms include decreases in Ca^{2+} uptake required for the actin-myosin coupling mechanism and change in endoplasmic reticulum functioning secondary to altered motor neuron activity and muscle activation. Because the soleus muscle is well established to be more sensitive to decreased neuromuscular activation (Roy and Acosta 1986; Roy, Talmadge et al. 1998), it is studied more extensively to explore the contractile properties of the skeletal muscle following SCI.

Similar to animal studies, chronically paralyzed individuals with complete SCI exhibit faster contractile properties of skeletal muscles. Depending upon the muscle studied, rates of force rise (contraction rates) have been reported to be significantly greater (+52%) and half-relaxation times are markedly shorter (-19% to 2fold) as compared to a healthy muscle. Similarly, contractile properties from chronically paralyzed upper extremity thenar muscles also show fast muscle characteristics (Thomas 1997). Note worthily, at relatively earlier time points after injury (within 6-24 weeks post SCI), contractile properties of the human paralyzed muscles are rather variable. Time to peak tension of the paralyzed soleus and quadriceps muscles, for example, has been demonstrated to remain the same or even slower than control values (Shields, Law et al. 1997; Castro, Apple et al. 2000). This disparity in the contractile properties of acute and chronic paralyzed muscles is attributed to the relationship between contractile speed and MHC isoform expression (Burnham, Martin et al. 1997; Dupont-Versteegden, Houle et al. 1998). At 6-24 weeks, fiber type conversion and hence MHC type expression has not occurred thereby retaining the contractile characteristics of the paralyzed muscle (Shields, Law et al. 1997; Castro, Apple et al. 1999). As far as the relaxation time is concerned, the half-relaxation times are slower

in the skeletal muscle of acutely injured subjects (-47%). Briefly, the rate of Ca^{+2} uptake or the rate of the cross-bridge detachment is surmised to have been compromised thereby slowing the overall muscle relaxation rates (Shields 2002).

Altered contractile properties of a paralyzed muscle have important functional implications. Paralyzed human muscle fatigues much more rapidly than a healthy muscle. Increased fatigability of skeletal muscle as characterized by force loss over repeated contractions with otherwise "non-fatigable" electrical stimulation parameters is a common phenomenon of chronically paralyzed slow muscles (Shields 2002). Declines in isometric force production over repetitive bouts of contraction are as large as 60% in the paralyzed skeletal muscle as compared to 40% declines in controls. Furthermore, muscle endurance, as measured by the muscle's ability to maintain force levels when stimulated repeatedly, is drastically reduced following SCI. While a healthy quadriceps muscle can maintain force levels of more than 30% of maximum isometric contraction for as long as 10 minutes, a paralyzed quadriceps muscle can maintain similar force levels for only less than 4 minutes (Gerrits, De Haan et al. 1999). Such weakness and fatigability of locomotor muscles potentially limits the use of rehabilitation programs that emphasize using functional electrical stimulation to achieve standing and walking.

1.4.4 Metabolic Adaptations: Altered Oxidative Capacity

Alteration in skeletal muscle mitochondrial oxidative capacity and subsequent impairments in endurance are reflected by a variety of muscle adaptations following SCI. Mitochondrial oxidative capacity is a function of mitochondrial volume, competence and oxygen delivery to the mitochondria (McCully, Mancini et al. 1999; Kemp, Roberts et al. 2001). It is commonly used as an estimate of oxidative energy supply to the muscle and reflects overall muscle endurance. Mitochondrial oxidative capacity is altered following SCI (Jiang, Roy et al. 1990; Jiang, Roy et al. 1990; Otis, Roy et al. 2004). Histochemical measurements of various oxidative enzyme

activities from muscle homogenates or biopsy samples serve as markers of muscle metabolic capacities. Additionally, non-invasive measurements of metabolic capacities by magnetic resonance spectroscopy have been well established and are found to significantly correlate with mitochondrial enzymes activity measured with biopsy specimens (McCully, Fielding et al. 1993).

Generally, marked alteration in muscle oxidative capacity is reported following SCI in humans. Martin et al and Rochester et al have shown that the chronically paralyzed human tibialis anterior muscle shows marked decrease in succinate dehydrogenase (SDH) activity as compared to able-bodied individuals (Martin, Stein et al. 1992; Rochester, Barron et al. 1995). Kjaer et al have demonstrated a marked decrease in activity of a variety of oxidative enzymes (almost 1.5 to two fold) from the vastus lateralis muscle of men who sustained a complete spinal cord injury for almost 12 years (Kjaer, Mohr et al. 2001). Hartkopp et al report a significantly lower oxidative capacity in the upper extremity wrist extensor muscles of persons with more than five years of SCI (Hartkopp, Harridge et al. 2003). An overall decrease in mitochondrial oxidative capacity, also measured by decreases in mitochondrial DNA content, (reflective of mitochondrial protein content), capillary density and blood flow are reported following chronic SCI in humans (Scelsi, Marchetti et al. 1982; Wang, Hiatt et al. 1999). Furthermore, as early as 11 weeks after complete SCI in humans, Gregory et al have shown decease in SDH enzyme activity by almost 41% (Gregory, Vandenborne et al. 2003). However, in a follow up study at 24 weeks after injury in the same subjects the SDH activity returned to control values (Castro, Apple et al. 1999).

Interestingly, oxidative enzyme activity in animal models of SCI is rather muscle specific. After six months of both spinal cord transection and spinal isolation, the cat soleus muscle either

enhances or maintains SDH activity in comparison to control rats implying maintenance of muscle oxidative potential after chronic SCI in the slow skeletal muscle (Jiang, Roy et al. 1990; Graham, Roy et al. 1992). Elevated SDH activity is also demonstrated in the spinalized rat soleus muscle 6 months after injury (Otis, Roy et al. 2004). Though mechanisms to elucidate the elevated oxidative enzyme activity are unknown, investigators have attributed the greater oxidative capacity in slow muscle to the presence of different fiber type composition of the paralyzed slow muscle. Apparently, the paralyzed slow soleus muscle possesses an unusual proportion of hybrid fibers (Roy, Talmadge et al. 1999) that in turn are purported to have inherently larger oxidative capacities than the typical type 1 muscle fibers (Rochester, Barron et al. 1995; Otis, Roy et al. 2004). In fact, mixed muscle such as the gastrocnemius and the vastus lateralis with predominantly fast muscle fibers (type IIx and IIb) show significant declines in SDH activity following spinalization in both cats and rats (Jiang, Roy et al. 1990; Gregory, Vandenborne et al. 2003). In an animal model of spinal transection, Durozard et al have used non-invasive magnetic resonance spectroscopy to demonstrate declines in oxidative capacity of the gastrocnemius muscle in rats. Based upon their data, the authors also suggest transition in source of energy supply from oxidative to anaerobic pathways for muscle metabolism following paralysis (Durozard, Gabrielle et al. 2000). Though change in enzyme activity is associated with phenotypic alterations following SCI (Martin, Stein et al. 1992; Rochester, Barron et al. 1995), change in muscle enzyme activities is also thought to occur independent of shifts in fiber types composition (Castro, Apple et al. 1999; Gregory, Vandenborne et al. 2003). Accordingly, the existence of any association between enzyme activity and fiber type alteration after SCI remains a subject for debate.

Irrespective of the cause, decrease in skeletal muscle oxidative capacity following muscle inactivity is purported to ultimately associate with sub-maximal endurance and exercise performance. Decrease in overall muscle endurance as measured by the fatigue index (defined as the decrease in force production with repeated muscle contraction) accompanies muscle inactivity following spinalization (Roy 1998, Shields 2002, Gerrits 1998). Researchers have partly attributed muscle fatigability following SCI to altered muscle oxidative capacity. This premise is somewhat supported by the observation that reduced physical training in able-bodied individuals causes significant declines in oxidative enzyme activity, which in turn drops long term skeletal muscle endurance (Houston 1979, Hickson 1982 from Kjaer 2001). Secondly, conversion to faster muscle fiber phenotype at the cost of slower muscle fibers predisposes the paralyzed muscle to acquire faster contractile speeds thereby making it easily fatigable (Shields, Law et al. 1997; Gerrits, De Haan et al. 1999; Hutchinson, Linderman et al. 2001). Lastly, studies have also linked reduced skeletal muscle oxidative capacity in the development of a major contributor of cardiovascular complications, namely altered insulin resistance (see below for more on insulin resistance) (Brehm, Krssak et al. 2006; Schrauwen-Hinderling, Kooi et al. 2007).

1.4.5 Metabolic Adaptations: Altered Glucose Homeostasis

Cardiovascular complications were the second leading cause of death in individuals with SCI until 1990 (Bravo, Guizar-Sahagun et al. 2004; Jacobs and Nash 2004). With increasing survival rates of SCI and the development of multiple cardiovascular risk factors, it is now the leading cause of death after chronic injury (Bravo, Guizar-Sahagun et al. 2004). Risk factors for these complications include development of insulin resistance, dyslipidemia (elevated low-density lipoproteins, depleted high-density lipoproteins and increase in total cholesterol), overall obesity and diabetes, a sedentary lifestyle and limited exercise (Bauman and Spungen 2001;

Bravo, Guizar-Sahagun et al. 2004; Jacobs and Nash 2004). Of these, glucose intolerance due to development of insulin resistance is suggested as the main cause of cardiovascular disease after SCI (Bravo, Guizar-Sahagun et al. 2004). Insulin resistance is the resistance of target cells such as muscle or liver tissue to insulin. The target cells (muscle and fat that take up glucose) are said to be resistant to glucose uptake and/or are less sensitive to the peripheral uptake of blood insulin. Elevated blood glucose and insulin levels subsequently precipitate as one of the earliest hallmarks in the development of type 2 diabetes mellitus (Jacob, Machann et al. 1999). In fact, persons with complete and incomplete SCI are predisposed to hyperinsulinemia and an imbalance in glucose homeostasis with subsequent development of insulin resistance (Bauman, Spungen et al. 1999; Bauman and Spungen 2001). Additional risk factors include relative increases in adiposity, a sedentary lifestyle and muscle atrophy. The association of skeletal muscle and insulin resistance is explained below.

Skeletal muscle tissue is responsible for the majority of the insulin mediated glucose disposal in the body (Goodpaster and Kelley 1998; Schrauwen-Hinderling, Hesselink et al. 2006). The study of intramuscular fat content has gained considerable attention because of the association of high levels of skeletal muscle lipid with insulin resistance. Existence of lipid in skeletal muscle was first identified by Denton and Randle in 1967 (Denton and Randle 1967). However, it is only since the last decade, that wide arrays of studies have reported associations between intramuscular fat and insulin resistance (Perseghin, Scifo et al. 1999; Furler, Poynten et al. 2001). Pan et al first established this relationship; they found that muscle triglyceride content correlates with insulin resistance irrespective of total body adiposity (Pan, Lillioja et al. 1997). Lipids are typically stored in the skeletal muscle in the form of intramyocellular lipid (IMCL) or extramyocellular lipid (EMCL). EMCL is the plate or tube shaped fatty infiltrate located outside

the myocyte and between muscle fibers (Szczepaniak, Babcock et al. 1999; Boesch, Machann et al. 2006). EMCL can also be located intramuscularly as adipose tissue along with fascia separating fascicles of the same muscle, inter-muscularly along with fasciae between adjacent muscles and in subcutaneous fat layers. EMCL is purported to be a long-term storage depository and suggested as being metabolically relatively inert. It is utilized for energy production during very low intensity exercises and has no known correlation with insulin resistance (Boesch, Slotboom et al. 1997). In contrast, electron microscopic studies have established IMCL to be the fat located within the cytoplasm of muscle cell close to the mitochondria (Boesch, Slotboom et al. 1997; Schrauwen-Hinderling, Hesselink et al. 2006). Because of its proximity to the mitochondria and its relatively larger content in oxidative fibers, IMCL is regarded as an energy source for mitochondrial fat oxidation during rest and long-term endurance activities (Krssak, Petersen et al. 2000; Boesch, Machann et al. 2006; Schrauwen-Hinderling, Hesselink et al. 2006). IMCL can be mobilized and utilized within hours of physical activity, recovers after several hours following exercise and finally reaches normal levels within days (Boesch, Machann et al. 2006). However, excessive accumulation of IMCL can have a negative impact on insulin signaling to induce insulin resistance (see below). A variety of studies have confirmed the correlation of IMCL with insulin resistance in healthy persons as well with obesity and Type 2 diabetes mellitus (Jacob, Machann et al. 1999; Kelley, Goodpaster et al. 1999; Krssak, Falk Petersen et al. 1999; Perseghin, Scifo et al. 1999; Sinha, Dufour et al. 2002; Goodpaster and Wolf 2004; Schrauwen-Hinderling, Hesselink et al. 2006). Investigators have reported moderate to strong associations of IMCL with several markers of coronary artery disease (interleukin -6, homocysteine) and pre-diabetes states (insulin, insulin resistance) (Krssak, Falk Petersen et al. 1999; Weiss, Dufour et al. 2003; White, Ferguson et al. 2006). Accordingly, IMCL content is

suggested to serve as a potential non-invasive marker of insulin resistance. However, one limitation of using IMCL to describe insulin resistance is that IMCL is largely influenced by a host of factors including age, gender, diet, obesity, physical inactivity, exercise, genetics, ethnicity as well as the muscles studied (Forouhi, Jenkinson et al. 1999; Sinha, Dufour et al. 2002; Goodpaster and Brown 2005; Stettler, Ith et al. 2005; Schrauwen-Hinderling, Hesselink et al. 2006; Boesch 2007). IMCL levels are reported to be elevated in older people compared to young (Cree, Newcomer et al. 2004), females than males (White, Ferguson et al. 2006), people with high fat diets compared to low fat diets (Stettler, Ith et al. 2005) and higher levels in obese as compared to lean persons (Sinha, Dufour et al. 2002). Additionally, lipid content varies from muscle to muscle with the slow soleus muscle, for example, showing almost three times the IMCL content as compared to the fast tibialis anterior in the same subject (Rico-Sanz, Thomas et al. 1999; Hwang, Pan et al. 2001; Vermathen, Kreis et al. 2004). Accordingly, large variations in IMCL continue to exist even in non-diseased healthy individuals, making it difficult to relate it with insulin resistance. These factors therefore make it important to have stringent inclusion criteria even for healthy controls in a study design. Nevertheless, studies have attempted to document inter and intra-subject variations in the IMCL content of more than one muscle, making it feasible to still conduct these measures and reliably relate them with insulin resistance (Boesch, Decombaz et al. 1999; Torriani, Thomas et al. 2005). Positive correlations between IMCL and insulin resistance have been reported in sedentary individuals irrespective of sex, body weight and physical fitness (Sinha, Dufour et al. 2002; White, Ferguson et al. 2006). Presently, the advent of new spectroscopic techniques continues to encourage investigators to explore the relationship between IMCL and insulin resistance. Accordingly, simple ratio

measurements that yield intramyocellular fat content has the potential to serve as an *in vivo* biomarker of insulin resistance, which might prove beneficial in a variety of patient populations.

In persons with either complete or incomplete SCI, a three to four fold increase in thigh intramuscular fat is reported as compared to able bodied adults (Elder, Apple et al. 2004; Gorgey and Dudley 2006). These studies also reported that their patient group had high plasma glucose and insulin levels that were positively correlated to intramuscular content. Moreover, a decrease in skeletal muscle mass in this patient population was inversely correlated with plasma glucose levels suggesting that skeletal muscle atrophy and intramuscular fat both contributed towards the decrease in insulin sensitivity. Taken together, it appears that persons with SCI may be predisposed to the development of this health related risk factor and may also be at a relatively greater risk for the development of type 2 diabetes.

In the present work, an attempt is made to quantify IMCL in the skeletal muscle of persons with incomplete SCI (Chapter 7).

Mechanisms linking decreased insulin sensitivity (or increases in insulin resistance) with elevated IMCL remain ambiguous, but a variety of factors, either singly or in combination have been surmised for this association.

An increased level of IMCL is a possible result of greater uptake of fatty acid in muscle (Hegarty, Furler et al. 2003; Hulver and Dohm 2004). Studies have found strong negative correlations between adiponectin protein and IMCL measures in both obese and non-obese adolescent and adult populations (Stefan, Vozarova et al. 2002). Adiponectin, a protein released from adipose tissue is suggested to maintain normal triglyceride levels in blood. Elevated IMCL content from the skeletal muscle is established as a strong predictor of adiponectin levels suggesting a potential role of the protein in the accumulation of triglycerides in skeletal muscle

tissue (Stefan, Vozarova et al. 2002; Weiss, Dufour et al. 2003). Elevated IMCL content, in turn, increases intracellular fatty acids and their derivatives. Intermediates of fatty acid metabolism at the intramyocellular level (triacylglycerols, diacylglycerol, ceramide and fatty acyl CoAs) inactivate insulin action by inhibition of specific steps in the insulin-signaling pathway that is normally responsible for glucose uptake in the myocyte (Itani, Ruderman et al. 2002; Hegarty, Furler et al. 2003; Hulver and Dohm 2004). Studies report that the elevated diacylglycerol and fatty acyl CoA levels secondary to increase in muscle triglyceride, increases the protein kinase C activity. This enzyme can directly inactivate insulin receptors for uptake of insulin in muscle. Similarly, elevated ceramide levels mediate inhibition of signaling pathways by release of specific enzymes (Hegarty, Furler et al. 2003; Hulver and Dohm 2004). Another proposed mechanism suggests that increase in IMCL content leads to a decrease in insulin receptor synthesis in the skeletal muscle. Interference with insulin receptor function ultimately leads to an increase in insulin resistance, which in turn stimulates hepatocytes to increase serum triglycerides and decrease serum HDL (White, Ferguson et al. 2006). Therefore it appears that accumulation of IMCL serves as a mediator rather than being a direct cause of decreased insulin action.

Collectively, decreased insulin action (uptake in muscle) is proposed as the major contributor of insulin resistance in the myocyte. Though elevated plasma fatty acid levels ultimately increase IMCL content, which can lead to the development of insulin resistance, the reverse route that insulin resistance can lead to an elevation in IMCL content cannot be ruled out.

1.5 Summary

With technological advancements in the efficient management of acute SCI, the proportion of and life expectancy of persons with an incomplete injury has considerably increased. Varying degrees of tissue sparing and muscle loading presents the incomplete SCI population with

variable degrees of paralysis and paresis and hence distinct degrees of functional limitations. A host of skeletal muscle adaptations including muscle atrophy, muscle fiber type conversion, declines in contractile function, predisposition to muscle injury, fatty tissue infiltration, altered oxidative capacity and glucose homeostasis are well established after complete SCI in animals and humans. Despite the obvious motor dysfunctions, physiological muscle adaptations following incomplete spinal cord injury are relatively unstudied. With the advent of new therapeutic strategies and advances in SCI research aimed at recovery of lost functions, it is imperative that the machinery for movement and locomotion remain intact. The overall objective of the present work was to elucidate muscle adaptations after incomplete spinal cord injury in humans and in the rat model of incomplete SCI.

CHAPTER 2
SPINAL CORD INJURY AND LOCOMOTOR TRAINING

2.1. Locomotor Function after SCI

Initial insult to the spinal cord profoundly impacts almost all biological systems of the body. Specifically, neuromusculoskeletal deficits following the injury tremendously impair the motor performance and locomotor capabilities of individuals with SCI. Following incomplete SCI; there always exists some degree of spontaneous recovery. As a result, persons with incomplete SCI exhibit variable paralysis and paresis of affected muscles, typically resulting in impaired motor performance and varying degrees of functional limitations (Subbarao 1991; Tang, Tuel et al. 1994; Burns, Golding et al. 1997). Similar to persons with complete-SCI, persons with incomplete-SCI exhibit a variety of clinically relevant motor and functional deficits, including local muscle fatigue, weakness of affected muscles (Sloan, Bremner et al. 1994; Johnston, Finson et al. 2003) and diminished capacity to ambulate (Waters, Adkins et al. 1994; Ulkar, Yavuzer et al. 2003). Other studies have shown a significant reduction in ambulatory capacity, with functional deficits in gait including a reduced gait speed, step frequency; stride length and longer durations spent in the double support phase of gait cycle (Melis, Torres-Moreno et al. 1999; van der Salm, Nene et al. 2005). Loss of ambulation is the most obvious functional limitation associated with a SCI and regaining walking capability one of the main aims of this patient cohort (Kilgore, Scherer et al. 2001). In a report by Iezzoni et al, "Walking holds profound symbolic importance. Nowadays, upright movement permeates American aphorisms, connoting independence, autonomy, self-reliance and strength". Inability to "walking tall" creates a physical need to restore mobility and an emotional need to restore one's "core sense of value and place in the world" (Iezzoni 1996). With the remarkable focus that is now spent for new treatments for SCI, a lot of energy is spent in exploring the

neuromuscular physiological impairments associated with locomotion (Dobkin 2000; Wolpaw and Tennissen 2001; Dobkin and Havton 2004; Fouad and Pearson 2004). This approach has in turn held optimism in determining mechanisms associated with other functional impairments seen in this patient population such as urination, sexual, and bowel function that at present are less understood. Over the past two decades, researchers in the field of SCI have come to recognize the remarkable potential of the central nervous system to regenerate following its injury. Accordingly, investigators are discovering new therapies to improve the locomotor capability of persons with SCI. The rest of this chapter includes a brief review of: a) Conventional rehabilitation for persons with SCI b) limitations of current clinical practice in SCI c) spinal cord plasticity and principles of neuroplasticity d) a potential paradigm shift in enhancing locomotor function after SCI e) locomotor training in animal and humans

2.1.1. Conventional Rehabilitation Therapies after SCI

Present rehabilitation goals in a clinical setup for individuals with SCI are re-entry into community, energy conservation and functional independence. To achieve these goals, much of the rehabilitation is focused on task-oriented training along with compensation of lost abilities (Thompson 2000; Dobkin and Havton 2004). Patients are trained to perform a functional task that is specific to the goal to be achieved, but at the same time, achievement of the task accompanies using spared residual function and/or modifications in a person's environment. Thus, patients use whatever residual sensorimotor functions persist after the injury along with use of assistive aids to maximize their self-care, mobility and community roles. These task specific strategies are also called compensatory techniques in current practice (Mathiowetz and Haugen 1994). Examples of compensatory strategies that are commonly seen and/or taught in clinical practice for individuals with SCI include use of assistive aids (example: orthoses/crutches/wheelchair), new movement strategies (example: hip hiking) (Bell 1955) and

electrical stimulation induced movements (Peckham, Keith et al. 2001), (Dobkin and Havton 2004).

Use of a wheelchair after a SCI is one of the most common compensatory strategies. A lower extremity motor score (LEMS) of 20/50 or less indicates that persons with SCI are likely to be limited ambulators (ASIA 2001). Clinically, a wheelchair prescription is a common choice for such persons. However, sometimes, wheelchair use stems from the need to enhance the mobility and independence in the community [for reference see (Minkel 2000).

Functional electrical stimulation (FES) is being used extensively in improving functional motor performance of persons with SCI. Generally, FES devices are designed to comprise of a control unit and stimulating electrodes. The control unit translates signals from sensors or a voluntary movement onto the stimulating electrodes that are taped over the skin or surgically implanted near the nerve for specific muscle contraction. Mild electrical stimulus elicits muscle contraction that translates into muscle movement. By adequate alterations in stimulus parameters of the FES device, persons with SCI gain assistance in movement initiation and control. FES has been used widely to facilitate a variety of functional activities following SCI including controlling upper extremity movements and grasps, improving cardiovascular conditioning and breathing, training to walk for short distances, standing up for transfers and controlling bowel and bladder function (Creasey, Grill et al. 2001; Johnston, Finson et al. 2003; IOM 2005). Physiological effects of FES involve increase in muscle mass, aerobic metabolism and maintenance of bone density (Postans, Hasler et al. 2004); which ultimately play an important role in prevention of musculoskeletal complications after SCI. However, the role of FES in improving the ambulatory capability after SCI is limited. Expense of the equipment, assistance needed for set up, time taken from other daily activities, and the meager daily effects of FES

exercise deter patients from its use (Dobkin and Havton 2004). The gait patterns with use of FES are unrefined and allow for short-distance ambulation. Moreover, two major deterrents to the use of FES for functional performance include a) presence of an optimal degree of initial motor and sensory control in the lower extremities and b) a relatively lower level of incomplete SCI (lower thoracic); thereby making it suitable for use for only a limited population of SCI. Therefore, FES devices for assisting in ambulation are limited in scope. Most importantly, though FES signals are proposed to alter central activity dependent plasticity, sensory feedback induced by FES is not likely to cause any persistent central sensori-motor activation or motor skills learning [for review see (Dobkin and Havton 2004). So ultimately, FES still represents a compensatory strategy that leads to non-use of the persons' neurological systems and added disability in the long-term still persists.

[Note: Though numerous other compensatory strategies concerning other body systems are commonly implemented in rehabilitation (5), in this discussion, the primary focus is made on techniques aimed at enhancing locomotor performance].

2.1.2 Limitations of Compensatory Rehabilitation Strategies

Compensatory techniques are focused little, if any on the neural processes necessary or neurophysiological adaptations occurring secondary to performance of a functional task. In fact, "there is no evidence that programs of rehabilitation have any effect on restoring impaired nervous system function or enhancing natural recovery following disease or injury" (McDowell 1994). It appears that by using compensatory strategies and provisions in the environment, one completely ignores what a person's body is capable or might be capable of doing. Any potential for the person to explore the capabilities of his or her body systems to regenerate or restore is completely neglected. Today community environments are becoming easily accessible for people using wheeled devices as their primary means of mobility. Though this approach definitely

permits successful mobility of individuals in the community, long-term effects of such therapy can precipitate further disability. In fact, impact of long-term use of a wheelchair causes further disuse and negative plasticity of the neuromuscular system, which in turn creates further dependence on the use of a wheelchair for mobility. As it turns out, such interdependence leads to a host of psychosocial and physiological consequences that further enhance disability.

For a therapist, wheelchair provision for his/her client might be perceived as an opportunity of mobility, but a young man's opinion about his wheelchair has been - "You might as well stick me in a damn closet. That wheelchair just makes me think of how hopeless I am" (Minkel 2000). Thus, psychologically, many people interpret dependence on assistive aids as an indication of greater disability rather than an option for increased mobility (Iezzoni 1996). Physiologically, inactivity/disuse of the affected body part leads to more systemic complications (muscle atrophy, more predispositions to fractures, contractures, etc) and further dependence on walking aids for mobility. Overcompensating with spared motor function limits the capacity of the central nervous system to adapt (Barbeau, Fung et al. 2002). As such, prolonged disuse conforms to the frequently used adage-"use it or lose it". Few studies also associate disuse with suppression of genes that attempt axonal regeneration (for reference see (Dobkin and Havton 2004).

2.2 Spinal Cord Plasticity

The main reason for use of compensatory techniques to recover function after SCI has evolved from the doctrine that the spinal cord is nothing more than a hard-wired system that simply serves as a conduit for ascending and descending axons. More than 3500 years ago, SCI was referred to as "A disease that cannot be treated" (from (Vikhanski 2001). This dogma dominated for several centuries. In fact, Ramon Cajal in 1906 was awarded the Nobel Prize for discovering the regeneration capabilities of peripheral nerves while simultaneously confirming

that “as is well known, the central tracts are incapable of repair” (Vikhanski 2001). A true paradigm shift in the field of spinal cord neuroscience occurred in the 1950s. Windle, Liu and Chambers first suggested “sprouting” (referred to as growth of short shoots or sprouts from healthy nerves to compensate for damaged fibers) of healthy nerves within the central nervous system as a mechanism of recovery in experimentally spinalized animals (Windle 1954; Liu and Chambers 1958). However, scientific proof for nerve repair and regeneration within the spinal cord emerged only in the 1980s (Richardson, McGuinness et al. 1980). In the past two decades, animal and human research has revealed the potential capability of the CNS to repair and regenerate following its injury (Dobkin 2000; Thompson 2000; Rosenzweig and McDonald 2004). Currently, convincing evidence from spinal cord repair literature suggests that the most important contributors inhibiting growth of neurons is the presence of an non-permissive environment that consists of a mechanical barrier such as a scar tissue, insufficient neurotrophic factors for axonal regeneration and the presence of inhibitory molecules within the scar tissue (Fouad and Pearson 2004; Rosenberg, Zai et al. 2005). In the field of SCI research, vigorous attempts have been made in understanding the basic mechanisms of neuronal cell growth, death and repair; influence of central and peripheral neural signals on motor neuronal output; identifying spinal pathways (such as the central pattern generators) for locomotor function and strategizing new therapeutic modalities based on understanding of these mechanism. Neurons within the spinal cord have a complex interaction with cortical neurons, sub-cortical neurons (neurons within brainstem), motor neurons and afferent pathways. Indeed, the spinal cord has a large potential for plasticity at multiple levels of its architecture including afferent neurons that enter the cord, inter-neuronal population interposed between these inputs and the motor neurons, ascending axons within the cord, descending axons within the cord, synaptic connections on the

motor neurons and motor neurons themselves. Furthermore, alterations in neurotransmitter and neuro-modulator activity along with cortical changes accompany SCI (Bregman, Coumans et al. 2002; Dobkin and Havton 2004).

Rightfully, the term 'neuroplasticity" has also been extended to the spinal cord. Neuroplasticity is a permanent change in the structure and function of the nervous system in response to experience, ones' state of health and disease and experimental manipulation or injury. It is important to note that persistent changes *both* in the peripheral and descending input to the cord via myriad of factors such as practice, trauma, and disease will cause plasticity at multiple levels of the nervous system - both spinal and supraspinal. Therefore, acquisition of any new behavior - whether it is skilled learned behavior through prolonged practice (or disuse) or an abnormal response associated with CNS disease or compensatory movements - is inevitably associated with neuroplasticity. Interestingly, the spinal cord is so flexible that spinal neuroplasticity is a function of experiences throughout one's subsequent life (Wolpaw and Tennissen 2001). In this section, major focus is made on a specific, activity-dependent plasticity (walking) of the spinal cord. Activity dependent plasticity is the lasting change that occurs in the spinal cord because of activity (sensorimotor or sensory inputs) from the periphery or the brain. Such a change finally affects output of the spinal cord. However, to retain neuroplasticity, few basic rules are essential for its expression. These rules, focused on physiological recovery following neural injury eventually form the fundamental principles of current neurorehabilitation techniques.

a) Repetition/practice: Repeated performance of a task is invariably associated with learning. Thus, practice makes perfect. Physiologically, learning is but a reorganization of neuronal mechanisms (Kandel 2001). Repetition stimulates multiple neurons and consequently

alters neural activity that persists for long durations even after the activity ceases. The physiological mechanisms associated with learning range from neuronal pre-synaptic and/or post-synaptic structural changes, modulation of neurotransmitters, expression of new proteins, activation of silent synapses, growth of new neural spines (Kandel; Kandel 2001) and changes in representation of the cortical homunculus (Byl and Melnick 1997; Byl 2003). Therefore, for such permanent physiological changes to manifest, the stimulus that causes it must be repetitive. Moreover, whether the repetitive experience is in the form of excess or diminished stimuli (example activity versus disuse), plasticity is inevitable. Several studies have demonstrated the critical role of repetition for neural plasticity (Kandel; Wolf and Segal 1996; Beaumont and Gardiner 2003). For example: single shock of tactile electrical stimulus to the aplysia-siphon gives rise to a memory lasting only minutes, while four or five spaced shocks gives rise to a memory lasting several days (Kandel 2001). Soleus H-reflex modulation in monkeys was demonstrated after 3000-6000 trials/day (Wolpaw 1987). Therefore, to promote learning of a new motor task or for retraining, tremendous emphasis is placed on repetition as one of the major treatment principles in neurorehabilitation research (Edgerton, Roy et al. 1992; Behrman, Lawless-Dixon et al. 2005).

b) Task specificity: The nervous system will respond to whatever stimulus it is exposed to. Specific sensory stimulus will stimulate plasticity of appropriate neuronal circuits. In fact, only the activated neural pathways will respond to the stimulus. Moreover, any motor output is generally associated with and/or is a result of precise sensory experience. For example: the normal pattern of walking requires afferent sensory stimulus from the feet (Rossignol, Chau et al. 1996; Dietz and Harkema 2004; Duysens, Bastiaanse et al. 2004). To enhance firing of specific neuronal circuits and hence strengthen their synaptic efficiency, the same activity needs to be

entrained. For example: spinalized cats that were trained to walk had faster walking speeds than animals that were trained to stand only (Hodgson, Roy et al. 1994). In fact, locomotor training that closely simulates normal walking pattern has been suggested to improve functional locomotor outcomes while conventional rehabilitation approaches have not shown similar effects of training (Barbeau, Norman et al. 1998; Dobkin BH 2003, Sep).

c) Pattern of practice: Whether stimulus of practice is continuous or intermittent clearly dictates plasticity. Generally, intermittent practice is a more appropriate stimulus than continuous under wide circumstances of plasticity. For example: intermittent and not continuous hypoxic exposure elicits long term enhancement of inspiratory motor output known as long term facilitation (LTF) (Baker and Mitchell 2000). Also, continuous sessions of tactile stimulus to the aplysia-siphon leads to a short-term memory for habituation of the gill-withdrawal reflex, while intermittent sessions produces a long-term memory (Kandel 2001). One of the probable reasons for this pattern is the molecular neural mechanism associated with plasticity. Mechanisms of phrenic motor neuron LTF for instance, involve intermittent release of serotonin, which in turn initiates a cascade of events for protein synthesis. The elevated protein increases synaptic strength and hence phrenic motor output (Baker-Herman and Mitchell 2002). In fact, continuous release of serotonin (due to continuous hypoxia) may have an inhibitory effect on the pre-synaptic receptors for protein synthesis, thereby hindering plasticity. Thus, based on findings in lower animals, it appears that interval training might be better preferred over massed practice.

2.3 Locomotor Training: A Paradigm Shift

With the above background in mind, one can deduce that rehabilitation techniques aimed at enhancing locomotor function following SCI should minimize the use of compensatory strategies. As such, a physiologically based intervention program seems to be a more scientific approach towards recovery. As is well recognized now, regaining neuromuscular functions

following a neurological injury largely depends upon recognizing the pathophysiology of injury. The current scientist's perspective of recovery based therapy dwells from the notion - "Much needs to be done in the management of neurological disability and it will only be through basic science clarifying the mechanisms of disability and the application of scientifically sound outcome measures that interventions will make a real difference" (Thompson 2000). Consequently, approaches for rehabilitation of the SCI cohort needs to be emphasized on task-specific training that can trigger appropriate neural mechanisms. When developing therapeutic interventions to enhance functional recovery after SCI, an understanding of the underlying physiology of neuromuscular responses that occur after this type of injury will promote different interventions that rely less heavily on compensatory rehabilitation. Locomotor training using treadmill and overhead body weight support (LT) is one such rehabilitation strategy that is based on the neuroplasticity principles of walking recovery and has gained tremendous momentum in improving walking ability of persons with SCI (see below). The LT approach has proved more beneficial than conventional therapy in chronic incomplete spinal cord injuries. The following section includes a brief historical perspective and description of LT in animals and in individuals with SCI.

2.3.1 A Historical Perspective and Locomotor Training in the Spinal Cord Injury Animal Model

One of the most remarkable finding in spinal cord injury rehabilitation research is that spinalized cats were able to step with their hind limbs on a moving treadmill. This phenomenon evolved from the pioneering works of Shurrager and Dykman who emphasized the importance of regular repetitive locomotor movements in spinalized mammals (Shurrager and Dykman 1951). Later in the 1970s, Grillner et al and Edgerton et al were able to demonstrate the ability of the isolated spinal cord to produce cyclic activity between agonist and antagonist muscle groups

with levels of coordination that mimicked normal locomotion (Grillner and Zangger 1975; Edgerton, Roy et al. 1992). Since then, numerous investigators have demonstrated the stepping phenomenon in spinalized mammals on a moving treadmill (Forssberg, Grillner et al. 1980; Lovely, Gregor et al. 1986; Barbeau and Rossignol 1987; Edgerton, Roy et al. 1992). A typical training protocol in the animal model involves walking of a thoracic spinal cord lesioned animal on a treadmill. Initially stepping is possible only with manual assistance by trainers that assist with stepping of the hind limbs and application of a non-specific sensory input from the perineum, abdomen or the tail. Generally, after three months of daily weight supported treadmill training, the amount of support required to step declines and hind limbs move into a more rhythmic alternate pattern that closely resembles normal walking (Edgerton, Roy et al. 1992). Comparative data show that trained spinalized cats show better ability to step than those that are allowed to recover spontaneously (de Leon, Hodgson et al. 1998). Stepping is also associated with recovery of EMG patterns in the paralyzed muscles close to the pre-spinalized patterns (Lovely, Gregor et al. 1990; Hodgson, Roy et al. 1994). Note worthily, training outcomes in incompletely injured cats differ from spinalized cats. Incompletely injured cats eventually recover voluntary quadrupedal locomotion overground and on the treadmill within three days to three weeks depending upon the severity of injury (Rossignol, Chau et al. 1996; Rossignol, Drew et al. 1999).

As mentioned in Section 1.3.1, the SCI rat model has gained enormous recognition for use in SCI research. Accordingly, though much of the basic concepts in the neuronal control of walking were established in the cat model, these findings have also been translated to the spinal rat model. Over the past few years, investigators have assessed the effects of treadmill training on the recovery of rats following spinal transection (Moshonkina, Avelev et al. 2002;

Moshonkina, Gilerovich et al. 2004) as well as contusion (Thota, Carlson et al. 2001; Multon, Franzen et al. 2003; Fouad and Pearson 2004; Stevens, Liu et al. 2006). In rats spinalized at the thoracic level as adults, nine weeks of LT (5days/week, 10minutes, starting on day one after operation) has been demonstrated to hasten hind limb joint movements in the spinalized rats, generate separate voluntary joint movements in the hind limbs and produce coordinated step-wise limb movements on a treadmill. This locomotor function has been shown to coincide with morphologically intact motor neurons in the spinal cord (Moshonkina, Avelev et al. 2002; Moshonkina, Gilerovich et al. 2004). In spinally contused rats, few studies in recent years have shown that LT improves overground hind limb locomotor function. Thota et al reported improvements in lower limb joint kinematics of spinally contused rats that led to recovery of coordinated locomotor function after 7 weeks, albeit with deformities in gait (Thota, Carlson et al. 2001). In 2003, Multon et al induced spinal contusion by compression and found significant functional gains in the trained animals throughout a training period of 9 weeks (30 min/day, 5/week). The training starting at 3 days post injury enabled the trained rats to voluntarily support their body weight at the end of training; while the untrained group showed spontaneous recovery just enough to move their hind limb joints (Multon, Franzen et al. 2003) Stevens et al in 2006 have observed the effects of a relatively shorter duration of LT (1 week, 20min/trial, 2trials/day) starting one week after spinal contusion in rats. An overall 32% in overall locomotor function (BBB score) was observed in the trained rats that correlated well with peak muscle force measurements (Stevens, Liu et al. 2006). Though LT has enhanced the functional locomotor capabilities after incomplete injuries, it is important to note that several factors including the severity of injury, dose of therapy, initiation of training, etc. can largely dictate the effect of LT in these incomplete injury models. Fouad et al for example, have shown

that LT (2*15min for 5 weeks) did not add to the locomotor functional improvements in the trained rats versus the untrained group. Even untrained contused rats show functional improvements as early as 7-14 days post training. However, compared to other studies of spinal contusion, this group utilized a different degree and kind of incomplete injury to the cord (relatively less severe dorsal incomplete transaction of the cord as versus cord contusion or compression). The authors suggested that spontaneous recovery in the control group probably curbed differences between the two groups and their findings could be attributed to either insufficient amount of training and/or insensitive testing outcome measures (Fouad, Metz et al. 2000).

Collectively, these findings suggest that LT enhances locomotor capabilities in spinal cord injured animal models. However, an important note to make is that though LT has shown to evoke stepping patterns in the mammalian animal model of complete SCI on the moving treadmill, studies have failed to demonstrate their translation into independent overground walking. Nevertheless, animal studies have laid the foundations for a potential new therapeutic strategy for locomotion recovery in humans with SCI and have yielded valuable information about spinal cord plasticity and its response to training.

2.3.2 Locomotor training in Individuals with Spinal Cord Injury

Based on the principles of locomotor training established in the animal model of SCI, the training has been transferred to humans with SCI. Barbeau et al performed one of the initial studies in humans to study the effects of locomotor therapy in individuals with SCI (Barbeau, Wainberg et al. 1987; Visintin and Barbeau 1989). Subsequently, various investigators worldwide demonstrated impact of LT in the walking capability of this patient population (Wernig and Muller 1992; Dietz, Colombo et al. 1995; Dobkin, Harkema et al. 1995; Harkema, Hurley et al. 1997; Behrman and Harkema 2000). Briefly, a typical LT protocol in humans

comprises of placing subjects on a treadmill while 0-50% of their body weight is supported by an overhead climbing harness. Therapists manually assist in stepping over the moving treadmill while attempting to maintain appropriate joint kinematics. Note that LT uses "body weight support" to support body weight during training; but the training involves utilizing numerous principles of neuroplasticity to enhance locomotion. The principles of LT, in which the stepping pattern is repeatedly trained while body weight support is provided, are based on basic science research demonstrating the role of the spinal cord in controlling locomotion in animal models of SCI. In humans, attempts are made to encompass several basic principles that facilitate the kinetic and kinematical parameters associated with phases of stepping. Briefly, these include: a) adequate limb loading on the stance limb; b) maintain appropriate body kinematics including appropriate joint angles and an upright and extended trunk and head; c) generate stepping speeds approximating normal walking speeds (0.75-1.25 m/s); d) coordinate timing of hip extension and unloading of limb in stance with simultaneous loading of the contra lateral limb; e) avoid weight bearing on the arms and facilitate reciprocal arm swing; f) aid symmetrical inter-limb coordination; and g) minimize sensory stimulation that would conflict with sensory information associated with locomotion. These principles not only facilitate balance control, but also provide the ensemble of appropriate input for motor output (Harkema, Hurley et al. 1997; Behrman and Harkema 2000; Behrman, Lawless-Dixon et al. 2005). Such an approach also ensures training consistency across researchers. Thus, LT is not to be considered as a modality, but is rather a training strategy that is based on neurophysiological principles derived from animal studies and whose implementation necessitates adequate professional training, knowledge and skill.

Though investigators have demonstrated EMG activity from the lower extremity muscles following LT in persons with complete SCI, unlike the animal spinalized model however, LT has

failed to demonstrate initiation of voluntary stepping pattern in humans with complete SCI (Dobkin, Harkema et al. 1995). Nevertheless, the training has shown significant improvements in the locomotor capabilities in selective populations of incomplete SCI. Accordingly; therapies directed towards recovery of walking function have focused extensively on the use of LT in persons with incomplete SCI (Wernig, Nanassy et al. 1998; Behrman, Lawless-Dixon et al. 2005; Behrman, Bowden et al. 2006). Considerable research efforts have been directed in revealing the major characteristics of the training, potential neuronal mechanisms associated with the training along with its potential role as a therapeutic strategy (Dobkin and Havton 2004; Hicks, Adams et al. 2005). Locomotor training has been suggested to have a positive impact on the walking ability, walking speed, kinematical parameters, distance walked, limb coordination, functional independence, ability to walk with fewer assistive aids, transition from use of a wheelchair to upright walking with assistive aids and subjective well being (Wernig and Muller 1992; Behrman and Harkema 2000; Hicks, Adams et al. 2005; Hannold, Young et al. 2006)). Locomotor training has also shown to induce modulation of H-reflex and EMG patterns towards close to control values that accompany improved walking capabilities (Dietz, Colombo et al. 1995; Trimble, Behrman et al. 2001). As a result of these behavioral and neurological benefits, the training has also been subject to a large multi-center randomized clinical trial – the SCILT (Spinal cord injury LT). This trial involved participation of around 140 persons with incomplete SCI with ASIA grades B, C or D within 8 weeks of injury. The experimental group received LT and the second group a similar intensity of standing and overground mobility training. However, no significant differences were observed in the primary outcome measures of walking speed, distance walked in 6 minutes and the functional independence measure scores for lower extremity (FIM-L) between the two groups (Dobkin, Apple et al. 2006). Nonetheless, LT has

shown tremendous potential as a therapeutic strategy in improving locomotor capabilities of persons with chronic incomplete SCI. In fact, LT has gained a new direction in the treatment of SCI in that: a) investigators are now proposing a strict patient selection criteria for conducting studies aimed at functional recovery of a heterogeneous population such as the incomplete SCI (Dobkin 2007) and b) various researchers are favoring combining LT with other training strategies for maximum locomotor gains. LT along with micro-stimulation of the spinal cord, pharmacological agents such as clonidine, and repair with nerve grafts are suggested as potential future strategies to enhance locomotor function after SCI (Herman, He et al. 2002; Dobkin and Havton 2004; Field-Fote 2004).

2.3.3 Central Pattern Generators and Locomotion

One of the principal neuronal recovery mechanisms promoting repair/regeneration of the injured spinal cord following LT is suggested to be the reestablishment of inter-neuronal connections known as the central pattern generators (CPG) within the spinal cord. The discovery of CPG in the quadruped spinal cord has stimulated much interest in current research on promoting neural regeneration following SCI. In quadrupeds, CPGs are established to exist as a group of inter-neuronal networks within the spinal cord that produce a rhythmic motor pattern resembling normal locomotion (Edgerton, Leon et al. 2001; Fouad and Pearson 2004). In spinalized cats, an approximate 25% of total daily-integrated EMG activity can be elicited from the soleus muscle during treadmill stepping; thereby implying the presence of a partial functioning spinal network even after a complete SCI. This fictive locomotion is produced independent of supraspinal and phasic afferent input (Grillner and Zangger 1975).

Though their presence is well known in rats and cats, presence of CPG in the human spinal cord is debatable. Based on the inability of persons with complete SCI to generate stepping, some investigators have refuted its presence in the human spinal cord. In contrast, support for the

existence of CPG in humans is well reported (Dimitrijevic, Gerasimenko et al. 1998; Lamb and Yang 2000; Dietz and Muller 2004; Edgerton, Tillakaratne et al. 2004). Dietz et al have demonstrated LT induced increase in leg muscle EMG activity when persons with complete SCI are made to step on a treadmill with body weight support and manual assistance (Dietz and Muller 2004). The authors suggest existence of neuronal networks within the spinal cord as the source of this enhanced neuronal activity. Furthermore, direct electrical stimulation of the lumbar spinal cord in humans with complete SCI has been shown to evoke locomotor-like rhythmic activity. This seemingly alternating pattern of the lower limbs has been attributed to the presence of a programmed inter-neuronal network within the spinal cord that via input from the electrical stimulation receives its "drive" to produce the motor output (Dimitrijevic, Gerasimenko et al. 1998). Lamb and Yang in 2000 further show that infants at birth are capable of stepping continuously when their feet are placed on a treadmill. Since infants do not have a functional descending spinal pathway, this locomotor behavior is attributed to a functional CPG within the spinal cord (Lamb and Yang 2000).

Note worthily, presence of some kind of sensory stimulation (peripherally or supra-spinally) presents as an essential prerequisite for mammalian locomotion. It is well known that descending pathways in the ventrolateral region of the spinal cord play a significant role in transmitting voluntary commands from the motor cortex to the spinal cord and are involved in initiation of locomotion (Noga, Kriellaars et al. 1991; Brustein and Rossignol 1998). These pathways most likely provide the stimulus necessary for CPG stimulation during normal walking. On the other hand, phasic afferent input has been demonstrated to play a key role for stepping in spinalized cats (Bouyer and Rossignol 2003), rats (Timoszyk, De Leon et al. 2002), as well as in humans with incomplete SCI (Dietz and Duysens 2000; Harkema 2001).

Investigators purport that one of the main sources of proprioceptive feedback during stepping is probably provided by the stretch sensitive and load sensitive receptors in the lower extremity muscles. While cutaneous receptors in animals are also influential in producing a motor pattern, they are implicated to play a larger role in skilled locomotor activities such as beam walking or paw placement on rungs of a horizontal ladder (Bouyer and Rossignol 2003).

Therefore, neuronal networks within the spinal cord (CPGs) can be regarded as autonomous; but sensory input is nevertheless necessary for both normal and spinal locomotion. In addition to peripheral input, the ensemble of supraspinal input is also necessary in the initiation, maintenance, balance and vestibular control of locomotion so as to adapt to environmental constraints. Locomotor function then is a consequence of neuronal interaction between a wide ensemble of information coming from supraspinal centers, afferent signals and CPGs (Edgerton, Tillakaratne et al. 2004).

2.4 Locomotor Training Effects on Paralyzed Skeletal Muscle

While locomotor training has proven to yield much neuronal plasticity, the following paragraphs discuss the effect of LT on skeletal muscle morphology and function in animal and human models of SCI. Locomotor training effects on the paralyzed skeletal muscle holds importance for two major reasons: 1) with increase in new therapeutic interventions for SCI, it is necessary that an intact machinery for limb movement is maintained; 2) current studies purport that exercise in normal rats increases the level of neurotrophic factors and proteins associated with neuronal growth and plasticity. Thus, exercise induced increase in neurotrophic factors produced in the muscle might, via their retrograde transfer, promote neurite outgrowth or synaptic plasticity within the injured spinal cord (Fouad and Pearson 2004; Hutchinson, Gomez-Pinilla et al. 2004).

Locomotor training has an overall ameliorating effect on the spinal transection or contusion induced muscle alterations. Generally, maximum effect of the training is seen in the slow extensor muscles of the lower extremities with minimal or no effect on the fast extensor or flexor muscles (Roy and Acosta 1986). Five weeks of daily locomotor stepping with emphasis on load bearing 30min/day, 5days/week, beginning one month after transection has shown to markedly alleviate the atrophic response in the lower extremity muscles of spinalized cats (Roy and Acosta 1986; Roy, Talmadge et al. 1998). In addition, a relatively higher proportion of Type 1a fibers are expressed in the paralyzed soleus muscle after LT as compared to before initiation of the training. Such conversion is reflective of transition to a healthier muscle. Furthermore, these studies show that the peak isometric forces produced by slow extensor muscles (soleus and vastus intermedius) increases to control values at the end of the training period. In addition, there is a concurrent increase in the overall oxidative enzyme activity after LT. Recently; Stevens et al have elucidated the impact of one week of LT, starting at 1week after contusion (20min/trial, 2 trials/day at 11mpm) on the skeletal muscles of contused rats. Significant increases in soleus muscle fiber cross-sectional area, peak tetanic force and decreases in muscle fatigue measurements have been demonstrated in trained rats versus untrained injured rats. Measurements of force improvement correlated well with functional performance (BBB score); implying marked improvement in motor recovery by LT of as short as one week (Stevens, Liu et al. 2006).

In humans with incomplete traumatic SCI (ASIA C), a couple of recent studies have reported the morphological and metabolic effects of LT on skeletal muscle. In nine persons with incomplete SCI, sixty-eight training sessions of LT spanned over six months increased the vastus lateralis fiber cross-sectional area, an overall increase in the expression of Type Ia muscle fibers

and increases in muscle oxidative capacity (Stewart, Tarnopolsky et al. 2004). These measures were accompanied by concurrent increases in the walking speed and locomotor capacities of injured subjects Subsequently, the same research group also observed increased vastus lateralis muscle glycogen stores (suggestive of increase in the glycogenolytic capacity of the muscle) and hexokinase enzyme activity (reflective of improvements in insulin sensitivity) in their subjects (Phillips, Stewart et al. 2004).

2.5 Summary

Persons with both complete and incomplete SCI display significant locomotor deficits following the injury. Current advances in the field of neuroscience and rehabilitation research are providing a new dimension to therapeutic approaches in SCI. Accordingly; physiological based restorative interventions have the tremendous potential to replace current conventional therapies. Locomotor training, based on the principles of neuroplasticity is one such intervention and has been used extensively to improve locomotor capabilities in select populations of incomplete SCI. In addition to alleviating various functional deficits, LT has the potential to induce several muscle adaptations including increases in muscle fiber size, fiber type conversion and improvements in muscle oxidative capacity. In the light of maintaining an intact peripheral machinery after SCI while also revealing potential effects of the trained muscular system on the neurological system, more studies are necessitated that focus on studying muscle adaptations following LT in persons with SCI.

CHAPTER 3
MAGNETIC RESONANCE AND SKELETAL MUSCLE

3.1 Introduction

Throughout the present work on both human and animal models of SCI, magnetic resonance (MR) is used as a key methodological tool to characterize lower extremity skeletal muscle. Skeletal muscle function depends both on its morphological and metabolic properties. Traditionally, muscle properties are studied using non-specific measurements (for example, anthropometric measures to determine muscle morphology), invasive techniques (for example, muscle biopsy to estimate fiber types or muscle enzyme profile) and/or global measures (for example, maximum oxygen consumption levels to reflect muscle oxidative capacity). In the past decade however, MR has gained tremendous momentum in characterizing skeletal muscle in healthy as well as a variety of patient populations. One of the most critical features of MR that makes it an extremely valuable tool in studying muscle is that it is non-invasive. In addition, the specificity, sensitivity and high resolution nature of MR makes it highly suitable for the assessment of a wide range of skeletal muscle characteristics including structural, functional and metabolic properties. Repetitive measurements enable users to determine disease progression and allow for follow up of various therapeutic interventions. Lastly, obtaining information about a physiological process from a functioning muscle in real time makes MR a unique non-invasive measurement technique in modern science. The following sections review the basics of MR, followed by distinct features of magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS) and their application in skeletal muscle.

3.2 Basics of Magnetic Resonance

The term Magnetic Resonance (MR) refers to the magnetic properties of the nucleus that is utilized in magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS).

MRI is an imaging tool that is capable of imaging soft tissues in the body; MRS, on the other hand, is used to study the metabolism of human tissues and organs.

Generally, a diagnostic image uses some physical property of the substance that is being imaged. For example, a photograph uses reflected light from the object that is pictured while ultrasonography uses reflected sound from the body part under study. Similarly, current MR techniques are based on receiving and processing signals from atomic protons. The exact molecular environment where these protons are located has a profound effect on the nature of the magnetic resonance signals created and thus gives rise to the remarkable power and versatility of MR. The following sections focus on the fundamentals of MR. However, it should be recognized that various complexities including nuclear spin physics theories and mathematical calculations that more completely explain MR are beyond the scope of this dissertation.

3.2.1 Magnetic Property of Nuclear Spins

An atom consists of a nucleus that contains positively charged protons and neutral neutrons. Surrounding the atomic nucleus is a cloud of negatively charged electrons that are located in orbits around this nucleus. In 1946, Nobel prize winners Felix Bloch and Edward Purcell theorized that any spinning charged particle (for example, specific charged atomic nuclei) when placed in a strong magnetic field creates an electromagnetic field around it (Bloch 1953)**.** The fact that these spinning particles behave as tiny bar magnets and can emit signal when subjected to a radiofrequency pulse has formed the basic concept of magnetic resonance (Figure 3-1).

Existence of a nuclear magnetic field depends on the number of unpaired protons in an atomic nucleus. According to quantum physics, protons in a nucleus are paired. For every proton spin with a magnetic field in one direction, a paired proton aligns in the opposite direction having an opposing magnetic field. Consequently, magnetic moments of a proton pair cancel

each other out and the net magnetic field is zero. In other words, when the number of protons in an atomic nucleus is even, the magnetic moments created by the paired protons cancel out each other and the net magnetic moment is zero. In contrast, when the number of protons is odd, there is always one proton that is unpaired and this gives rise to a net magnetic field or non-zero magnetic dipole moment (MDM) in the nucleus. The unpaired protons of an element can have their nuclear MDM oriented in either a parallel or anti-parallel direction. This alignment follows the Boltzmann distribution e-ΔE/kT (k stands for the Boltzmann constant) such that, at thermal equilibrium, part of the nuclei aligns anti-parallel (higher energy state), and a larger part aligns parallel (lower energy state). The net magnetization of a sample is equal to the sum of these individual nuclear magnetic moments. Generally, a MDM exists in any nucleus that has an odd number of protons. Nuclei of certain elements like Hydrogen (^{1}H), Phosphorus (^{31}P), Flourine (^{19}F), Sodium (^{23}Na), Carbon (^{13}C), Nitrogen isotopes (^{14}N and ^{15}N), Deutrium (^{2}D) and Oxygen isotope (^{17}O) have a MDM property (Garlick and Maisey 1992; Andrew 1994; Kevin K McCully 1994). Each of these nuclei can be used for MR purposes, but we use hydrogen atom for most MRI purposes and hydrogen, phosphorus and carbon for most MRS purposes. Hydrogen is the simplest and the most abundant element in the human body, since almost 60% of the human body is made up of water. Every water molecule has two hydrogen atoms and larger biological molecules such as lipids and proteins contain many hydrogen atoms. Sometimes enrichment (adding an extra proton to the nucleus) of nuclei is required to enable them for use in MR. Thus, enriching the ^{12}C atom to ^{13}C does not alter the chemical properties of the carbon atom much, but enrichment gives it a nuclear magnetic moment (Mulkern and Chung 2000).

3.2.2 Larmor Frequency

Each proton behaves like a bar magnet and has its own magnetic axis. In nature, the orientation of these axes is random. In the presence of a magnetic field (Bo) however, the axes

are aligned parallel to the axis of the main magnetic field. Spinning of protons around this axis when placed in a magnetic field is called precession. The frequency at which the protons precess in the magnetic field is directly proportional to the strength of the main magnetic field. This frequency is called the Larmor or precessional frequency. The Larmor equation expresses the relationship between the precessional frequency and magnetic field strength.

$$\mu=\delta \times Bo$$

Where μ is the Larmor frequency, Bo is the magnetic field strength and δ is the gyro magnetic constant. The gyro magnetic constant is a number without units that describes an intrinsic characteristic of a nucleus in a given environment. For water protons, the gyro magnetic constant is 42.6. At 0.5T the resonant frequency of water protons is therefore 21.3 MHz and at 1.5T it is around 63.9MHz. The gyro magnetic constant of protons within lipids and other non-water molecules is somewhat different. Thereby, the resonant frequencies of protons in different chemical environments are close, but not identical to that of water protons.

3.2.3 Longitudinal and Transverse Magnetizations

At the core of all MRI instruments is a homogenous magnetic field (Bo). The purpose of the magnetic field is to cause magnetization of protons within it. The precessing of protons gives rise to small secondary magnetic fields, or magnetization. The average magnetization of protons at a given time is referred to as net magnetization. At equilibrium, protons precess with their net magnetization align longitudinally along the axis of the main magnetic field and therefore the magnetization is more precisely referred to as net longitudinal magnetization, Mz.

This equilibrium/net longitudinal magnetization (Mz) can be considered as potential energy. Strength of Mz from tissues is dwarfed by the strength of main magnetic field Bo**.** Since we can only transmit and receive signals that oscillate, and the longitudinal magnetization is not an oscillating function, a receiver cannot read it. This severely limits detection of signal from

tissue protons at equilibrium. To detect MR signals for imaging, it is therefore necessary to disturb this equilibrium (Ray H Hashemi 1997). An excitation pulse, called the radio-frequency (RF) pulse is used to excite protons to emit radiations and cause disequilibrium. MR electromagnetic radiations are called RF waves and the pulse is called RF pulse because these are low frequency waves in the range of frequencies typically used by radio stations. These low frequencies are not capable of any DNA or biological tissue damage that is otherwise associated with high frequency ionizing radiations like X-Rays; a unique feature of MR that makes it exceptionally useful for use in living tissues. Note worthily, to excite a proton precessing within a magnetic field, the frequency of the RF pulse has to be similar to the precession frequency at which the proton is precessing. A resonance condition is one in which "energy may be transferred to and from a system very efficiently under unique conditions" (Gift, Pera et al. 1989). In absence of the unique conditions, energy transfer does not occur. Nuclear magnetic resonance in particular, involves measurement of signals coming from the atomic nuclei in response to radio waves that have the same natural frequency (precessional frequency) as the nuclei themselves. That is, nuclei may absorb energy in the form of electromagnetic waves from the RF pulse and give rise to a signal only when the frequency of the radio pulse exactly matches the nuclear magnetic moment precessional frequency. The Larmor or precessional frequency is called the resonance frequency because it is equal to the frequency of the radio pulse that induces this resonance (reverberations/echoes) in the protons.

Application of the radio pulse causes the net longitudinal magnetization Mz to rotate into the transverse plane, producing transverse magnetization Mxy (Figure 3-2). Since the transverse magnetization continues to precess in the x-y plane and is not obscured by the longitudinal magnetization of the main magnetic field, it is now capable of inducing current in a coil placed in

its vicinity. The coil is called the RF receiver coil and the induced current in the coil (i.e. the signal) is called an echo. Echo amplitude is greatest when it is first created. Once the excitation pulse is switched off, the amplitude of this signal rapidly decays and becomes weaker with time. Thus, the final signal is an oscillating decaying signal and is called free induction decay (FID). This signal is recorded and stored in the computer to construct an image.

3.2.4 Relaxation Times

Immediately after application of the RF pulse, Mxy precesses in the x-y plane around the z-axis. This happens only as long as the pulse is on. As soon as the RF pulse is switched off, the protons tend to regain their state of equilibrium, and hence their lowest energy state, by realigning themselves in the direction of the main magnetic field, Bo. The net axis of proton magnetization, Mz, eventually returns back to equilibrium. This return to equilibrium is referred to as relaxation. Relaxation is the process in which the spins are relaxing back towards their equilibrium state in the direction of the main magnetic field. Explanations of the two relaxation times associated with MR follow.

Longitudinal relaxation time (T1 relaxation time): T1 or longitudinal relaxation time is the time it takes to regain the longitudinal magnetization (Figure 3-3). As shown in the figure, at time T1, 63% of Mz is regained. That is, T1 relaxation time can also be referred to as the time required for the longitudinal magnetization to reach 63% of its original equilibrium level after complete flip by a 90° pulse. T1 relaxation rate (1/T1) is the rate at which the longitudinal magnetization Mz recovers along the z-axis after saturation by the RF pulse. Also, note that at time 2*T1, the longitudinal magnetization has recovered to 91% of its original equilibrium value and after three relaxation times, Mz has recovered to 97% of its original net magnetization (Mitchell 1999). T1 is also called the spin-lattice relaxation time because it refers to the energy that the protons have to give away to their surrounding (lattice) before gaining the equilibrium

state. Rapid transfer of energy to the surrounding lattice results in a shorter T1 and slow energy transfer to the lattice produces a long T1.

Transverse relaxation time (T2 relaxation time): As soon as the RF pulse is switched off, protons spin out of phase resulting in a rapid decay of the transverse magnetization Mxy. T2 relaxation time is the time it takes for the Mxy component to decay. Thus, in addition to T1 relaxation, a simultaneous but separate process is happening after the RF pulse (Gift, Pera et al. 1989). Mxy decay is a result of dephasing of protons once the RF pulse is removed. Dephasing occurs because of a) spin-spin interaction: because of their proximity, protons within the same molecule and between two molecules interact with each other. The spins dephase because of the consequent spin-spin interactions; b) external magnetic field inhomogeneity: inhomogeneties in the main magnetic field causes the protons to spin at slightly different frequencies.

T2 relaxation rate is the rate of decay of the transverse magnetization Mxy. T2 relaxation time can also be referred to as the time required for the transverse magnetization to decay to 37% of its signal (Figure 3-4). Like T1, T2 relaxation depends on inherent properties of the tissue and is fixed for a specific tissue. T2 of a tissue depends on how fast the proton spins in the tissue dephase. Rapid spin dephasing leads to a short T2 and slow dephasing causes a long T2. Tables 3-1 and 3-2 give a brief summary of the T1 and T2 relaxation times of human skeletal muscle (bound water), muscle lipid, intramyocellular lipids (IMCL) and extramyocellular lipids (EMCL) at various magnetic field strengths. Note that at a specific magnetic field strength, the T2 relaxation times of a tissue are 5-10 times smaller than the T1 relaxation times (Boesch 1999). Moreover, both T1 and T2 are affected by magnetic field strengths such that T1 gets longer and T2 decreases with increase in magnetic field strength).

3.2.5 Fourier Transform

Fourier transform is an extremely important and useful tool in MR that was introduced by Jean Baptiste Fourier. The MR signal is acquired in the time domain i.e. a waveform that varies with time. Hence, every signal is composed of a series of frequencies. The RF receiver coil detects and records not one Larmor frequency, but sum of all the possible Larmor frequencies produced from the frequency encoding gradients. To disentangle frequencies in the signal and therefore determine precessional frequencies of nuclear spins in the x and y directions, a mathematical manipulation of the signal called the Fourier transform (FT) is performed. FT basically decomposes arbitrary signals of time into familiar sine and cosine waves. In MR, FT enables one to determine which Larmor frequencies are present in any signal (Figure 3-5). Fourier transform of the digitized MRI data is the final image and that of the raw MRS data the final spectrum.

3.3 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is a non-invasive, non-ionizing and powerful imaging tool that is capable of imaging soft tissues in the body and is now being proposed as the imaging modality of first choice for a wide array of diseases. MRI can produce contrasts between soft tissues with great resolution. Contrast on MR images is a direct result of the different relaxation times of protons in body tissues. Though these times *per se* cannot be altered, two important MR parameters – repetition time (TR) and echo time (TE) enable tissue contrast. Appropriate adjustments of TR and TE allow putting more *weight* on T1 or T2 relaxation times of a tissue thereby yielding the most common T1 weighted, T2 weighted and proton density weighted (PDW) images. The choice of imaging is based upon the tissue desired for study.

3.3.1 T1 and T2 Weighted Images

Figure 3-6 shows T1 relaxation times of two tissues, one with a long T1 relaxation time and the other with a short T1 relaxation time**.** When the repetition time (time between two RF pulses; TR) is short, then the Mz component of the tissue with long T1 time possesses a smaller magnitude of recovery as compared to Mz component of tissue with short T1. Consequently, the magnitude of Mxy will differ largely between two tissues show larger differences in signal intensities. It is however necessary that the TR should be at least close or similar to the T1 of one of the tissues. This allows for best contrast between the two tissues. Also, a very short TR will not produce any signal because of insufficient recovery of the Mz component of either tissue. On the other hand, a very long TR will allow close to complete relaxation of both tissues such that their signal intensities will be similar (not shown here). A long TR therefore prevents differentiation between the two tissues. In this way, based on T1 of a tissue, tissue contrast is obtained using an appropriate TR. Because T1 of tissues dictates final image contrast, such images are called T1 weighted images. TE for such images is usually short.

Figure 3-7 shows T2 relaxation times of two tissues, one with a long T2 relaxation time and one with a short T2. After the Mz component is flipped to the transverse plane, spin dephasing of both tissue occurs. The tissue with long T2 will take longer to dephase as compared to tissue with a short T2. When images are acquired at an optimal TE, adequate differences in signal intensity exist between the two tissues. Therefore, differences in T2 relaxation will produce contrast between two tissues if the signal is collected at an appropriate TE. When TE is too short, the difference in SI is not much. For best T2 contrast, a TE is chosen that balances sufficient decay of transverse magnetization from one tissue against adequate presence of transverse magnetization from another tissue. Generally, images with TEs that approximate T2 relaxation times of tissues of interest have a significant T2 weighting. Lastly, very long TEs will

cause almost complete decay of transverse magnetization of both tissues and the ratios of their SI and hence contrasts between the two tissues are lost. In this way, based on T2 of a tissue, tissue contrast is obtained using an appropriate TE. Because T2 of tissues dictates final image contrast, such images are called T2 weighted images. TR and TE for such images are usually long.

Lastly, consider a sequence utilizing a long TR and short TE that yields a proton density image (Figure 3-7). When the TE is too short, enough time is not allowed for spin dephasing and the differences in signal intensity are purely based on differences in proton concentration of tissues. The T2 relaxation property of the tissue in this case is not explored. Furthermore, the long TR eliminates differences in contrast based on T1 relaxation rates of tissues. This is a proton density weighted image (PDW). For PDW image, one eliminates T1 effect by using a long TR and eliminates a T2 effect by using a short TE (The two factors that determine the respective image weighting). In such an image water will show up bright and fat will be less bright.

3.3.2 Image Construction

MR signal, like other radio waves, does not possess any directional information. Therefore, signals received contain information from the entire part of the body imaged. The fundamental process used to determine the location of the sources of MR signal and hence identify the specific body part imaged, is by application of magnetic field gradients. In a homogeneous magnetic field, water protons resonate at the same frequency, regardless of location. If a second magnetic field is now superimposed upon the main magnetic field, a predictable variation is observed in the magnetic field along a predetermined axis. The resulting magnetic field is highest at one end of the gradient and lowest at the other; between are intermediate values along the axis of the gradient. Thus, protons at one end of the gradient spin slower and protons at the other end spin faster. Gradients therefore create temporary

inhomogeneties in the main magnetic field to obtain spatial information. These signals from the protons can now be measured and used to construct images. The three basic steps in an imaging process are discussed below:

Slice selection*:* This process involves use of gradient during application of an excitation or refocusing pulse. A slice is selected by simultaneous application of a selective RF-pulse and gradient along the z-axis (conventionally defined) such that alignment of protons in a specific width of tissue is disturbed. The pulse excites certain portion of tissue that has the same resonant frequency as the gradient. At the end of the brief RF pulse, specific spins that are exposed to a specific gradient in a sample are excited. Magnetization vectors representing out-of-phase locations remain undisturbed and therefore do not contribute to the signal following the slice-selective excitation process. Application of a gradient causes tipping of protons only in a specific slice (Figure 3-8). The RF pulse has a specific frequency (called the central frequency) and a range of frequencies around this central frequency (bandwidth of frequencies) that excites a specific slice (as described above). The read out phase takes approximately 3msec. Though the signal is obtained from the entire slice, the slice image is not yet seen because of lack of in-plane spatial information about the slice. The signals are phase and frequency encoded for this purpose(Gift, Pera et al. 1989; Ray H Hashemi 1997; Mitchell 1999; Mulkern and Chung 2000).

Phase encoding: Phase encoding conventionally defines application of magnetic field gradients and hence image construction in the y-direction. To get spatial information in the y-direction, a gradient is applied in this direction. The phase encoding gradient (Bruhn, Frahm et al.) is turned on before application of the frequency encoding gradient (Gx) (explained next). It is usually applied right after the RF pulse or just before the Gx gradient or anywhere in between. Consider three rows of spins as in Figure 3-9. The left panel shows the spins before application

of Gy and the right panel shows the spins after application of Gy (arrows depict spin phases). After application of Gy, spins in the upper row experience a higher magnetic field and precess faster, spins in the lowest row experience the least magnetic field and precess slower. Spins in the center row experience no change in magnetic field and therefore do not change their precessional frequencies. Consequently, after a short time, protons in the three rows are left with different phases of their spins. When the Gy gradient is switched off, the precessional frequency of protons in each row becomes the same. However, the gradient has brought about a permanent shift in the spin phases. So the protons precess at same frequencies but are out of phase from each other. Because the gradient brings about a change in the phase of proton spins this process of image construction is termed "phase encoding". Note that a separate phase encoding step is needed for each row of pixels that needs to be discriminated in the slice. Therefore to discriminate between 256 rows in a slice, the process has to be repeated 256 times, each with a unique Gy gradient (phase shift).

Frequency encoding: Frequency encoding conventionally defines application of magnetic field gradients and hence image construction in the x-direction. This process occurs after the application of Gy and it is during this cycle that the signal is recorded as a function of time and stored in a computer. Consider the same matrix as defined above. To get spatial information in the x-direction of this matrix, a gradient is applied in the x direction called the frequency-encoding gradient (Gx). The left panel shows the spins before application of Gx and the right panel shows the spins after application of Gx. Note that application of Gy had already caused a phase shift. Application of Gx gradient varies the Larmor frequency of the spins in the three columns so that the spins in the right column have a higher frequency than the spins in the left column. Therefore, the central column of spins appears not to precess at all, the one on the right

has the highest frequency and the one on the left has the smallest frequency. In addition, because of the phase shift, each cell in the matrix ultimately experiences a unique phase shift and frequency. The read out phase takes approximately 10msec. The different frequencies are summed to form a signal that is detected by the receiver coil. The signal amplitude is a sum of all the frequencies and is recorded as a function of time. Note that the signal is collected only *once* during the read-out phase. That is, after one RF pulse, the Gy gradient is applied. This is then followed by the Gx gradient and subsequent measurement of the signal. Each signal is a representation of the entire image. However, a single RF pulse and hence one signal is not enough to produce the image. To get spatial information from body-tissue a series of RF pulses and phase encoding steps are necessary to resolve the image (Gift, Pera et al. 1989; Ray H Hashemi 1997; Mitchell 1999; Mulkern and Chung 2000).

3.4 MRI Applications in Skeletal Muscle

Skeletal muscle function depends on the morphological and metabolic properties of the muscle. MRI has the distinct ability to provide high-resolution spatial information and possesses the ability to specifically measure muscle size, quantify muscle damage, and visualize muscle recruitment patterns and intramuscular fat. The following paragraphs discuss the present and potential role of MRI in characterizing skeletal muscle.

Muscle size significantly impacts muscle function (Berg, Dudley et al. 1991; Ploutz-Snyder, Tesch et al. 1995; Stevens, Walter et al. 2004). Muscle size measurements include anatomical and physiological cross-sectional area (CSA), muscle thickness and length, and muscle volume. Traditional methods of assessing muscle size involve using anthropometric measurements like skin fold thickness and limb circumference, ultrasonography and dual X-Ray absorptiometry (DEXA). These measurements however, do not clearly differentiate between muscle and non-muscle tissue. Consequently, inclusion of non-muscle tissue like intramuscular

fat and connective tissue in muscle size measurements can overestimate muscle size. Other shortcomings include use of ionizing radiations (DEXA), limited field of view (ultrasonography) and inability to distinguish between individual muscles and/or muscle groups (anthropometric measures). MRI circumvents many of these disadvantages. MRI is non-ionizing, can clearly contrast between fat and muscle tissue, differentiates between contractile and non-contractile tissues within a muscle and can image the entire length of more than one muscle at the same time (Boesch 1999; Mulkern and Chung 2000). These advantages have established MRI as a gold standard of assessing skeletal muscle size. Numerous studies have used MRI as a standard technique to assess skeletal muscle size following disease processes (Mulkern and Chung 2000) , progression of muscle atrophy following disuse (Vandenborne, Elliott et al. 1998; Kitahara, Hamaoka et al. 2003) and effects of interventions (Stevens, Pathare et al. 2006). Moreover, anatomical and physiological CSA measures by MRI have been shown to correlate significantly higher with muscle strength measures such as maximum voluntary contraction than anthropometric and DEXA indices (Bamman, Newcomer et al. 2000).

Muscle T2 relaxation properties are sensitive to muscle contraction and therefore possess widespread applications in studying muscle characteristics. One of the initial studies reporting exercise induced increase in signal intensity of skeletal muscle T2 in humans was conducted by Fleckenstein in 1988 (Fleckenstein, Canby et al. 1988) . Fleckenstein et al showed that depending upon exercise intensity, contraction induced increase in T2 relaxation times can occur as early as after two muscle contractions, reach a plateau by few contractions and return to baseline values after 10-40 minutes of exercise. Interestingly, studies have successfully demonstrated positive correlations between elevated T2 times following exercise with integrated EMG patterns (Adams, Duvoisin et al. 1992). Furthermore, the exercise induced contrast

increases with exercise intensity so that the active area of the muscle is "mapped" to detect muscle use (Ploutz-Snyder, Tesch et al. 1995). Consequently, if a muscle shows more contrast shift, it reflects more use of the muscle and vice versa. (Adams, Duvoisin et al. 1992; Fleckenstein, Watumull et al. 1993; Ploutz-Snyder, Tesch et al. 1995). This phenomenon of muscle T2 has been used to infer which muscles are used during activity, the extent of their contribution, presence of any substituted activity by other muscles; including contribution of multiple and deep anatomical muscle groups at the same time. Thus, T2 changes are sensitive to the activity status of the muscle and therefore hold tremendous potential in identifying muscle activation patterns in both normal and diseased conditions.

Currently, studies indicate that the mechanisms of change in muscle T2 following rest to work transitions are a consequence of increase in T2 relaxation times of muscle water (Saab, Thompson et al. 2000; Patten, Meyer et al. 2003). This is probably a consequence of osmotically driven shifts of water into intramyocellular spaces secondary to accumulation of end products of muscle metabolism and/or intracellular acidosis (Ploutz-Snyder, Nyren et al. 1997; Vandenborne, Walter et al. 2000; Patten, Meyer et al. 2003).

Note worthily, the increase in T2 values after normal activity is transient and typically resolves within minutes to a couple of hours (Fleckenstein, Canby et al. 1988; Hayashi, Hanakawa et al. 1998). However, enhancement of and subsequent persistence of T2 values for longer periods (as long as two to three months) may indicate muscle damage. This is commonly observed following eccentric exercise protocols. Several studies have reported that eccentric exercise-induced muscle injury is associated with marked elevations in T2 values in both healthy and patient populations (Ploutz-Snyder, Tesch et al. 1995; Ploutz-Snyder, Nyren et al. 1997; Bickel, Slade et al. 2004). Elevated T2 values following strenuous exercises correlate with

specific markers of muscle damage including serum creatine kinase activity, plasma myosin heavy chain fragments (a specific marker of slow fiber muscle damage), muscle soreness and maximal isometric force (Foley, Jayaraman et al. 1999; Sorichter, Mair et al. 2001). Repeated MRI measurements of skeletal muscle for as long as two to three months following the exercise continue to show elevated T2 relaxation times; thereby suggestive of a long lasting change in the muscle (damage). Interestingly, similar long lasting elevated T2 relaxation times reflective of muscle damage have also been reported following reloading after hind limb immobilization, spaceflight and disease processes in both humans and animals (Bryan, Reisch et al. 1998; LeBlanc, Lin et al. 2000; Frimel, Walter et al. 2005). Muscle damage following reloading is attributed to mechanical disruption of muscle fibers and inflammation in muscle (Kasper, Talbot et al. 2002). Frimel et al have shown strong correlations between the elevated T2 relaxation times and histological markers of muscle damage (Frimel, Walter et al. 2005). Relatively large areas of T2 signal contrast have also been observed in the quadriceps muscle of persons with SCI as compared to control subjects after electrically stimulated muscle contraction. The authors in this study suggest that the increased recruitment of muscle in the SCI group not only implies more use of the muscle to evoke force; but also that persistence of the elevated T2 values most likely implies muscle damage (Slade, Bickel et al. 2004). In a follow up study, the same research group found that the thigh muscle areas showed a similar enhancement of signal intensities after a repeated bout of exercise that was followed 8 weeks after the initial bout. Since the controls did not show similar areas of elevated pixel signal intensities, the authors surmised that the skeletal muscle of persons with SCI remained injured and that probably the paralyzed muscles did not develop a protective effect following the first bout of exercise (Bickel, Slade et al. 2004).

Lastly, based on the high-resolution feature and ability of MRI to contrast between tissues; soft tissues within the muscle can be well demarcated using distinct MRI techniques. Accordingly, MRI has served as a non-invasive measure to identify, quantify and monitor progression of fatty tissue infiltration within skeletal muscle during diseased conditions (Huang, Majumdar et al. 1994; Elder, Apple et al. 2004). However, similar to other imaging techniques, T_2 weighted MRI also suffers from partial volume filling, making it challenging to reliably assess the inherent T_2 of skeletal muscle in the presence of increased amounts of intramuscular lipid.

Since elevated T2 values can reflect a variety of muscle processes including damage (Bryan, Reisch et al. 1998; LeBlanc, Lin et al. 2000; Frimel, Walter et al. 2005), edema (Ploutz-Snyder, Nyren et al. 1997; Patten, Meyer et al. 2003), fibrosis and fat (Huang, Majumdar et al. 1994), investigators are now making attempts to discriminate between these physiological processes utilizing a variety of advanced MR techniques including diffusion weighted imaging, magnetization transfer contrast and spectroscopy. Thus, the scope of MR in studying skeletal muscle is on a constant rise.

3.5 Magnetic Resonance Spectroscopy

While MRI provides spatial information, magnetic resonance spectroscopy (MRS) has been used for more than two decades to study the metabolism of human tissues and organs (Heerschap, Houtman et al. 1999). MRS is used to identify metabolites and monitor the absolute and relative concentrations of metabolites; thereby providing a non-invasive measure of physiology and pathology in body tissues.

3.5.1 Contrasting MRI and MRS

The main difference between MRI and MRS is that MRS does not result in images with spatial information, but rather results in a set of spectral peaks. The frequency encoding gradient

(read out) during MRI serves as a giant chemical shift in spatially discriminating proton spins along the conventional x-axis (Boesch 1999). In MRS, chemical shift is produced by the inherent nature of metabolites. Such inherent nature of the metabolites such as water and fat represent an artifact and spatial mis-registration in MRI. For example: artifacts at organ fat interface in the abdomen, vertebral body and intervertebral disc interface, bone muscle interface etc. Secondly, MRI uses the protons in the body's water molecules that have a concentration of 110M to obtain information about anatomy and pathology (Garlick and Maisey 1992; Boesch 1999). The sample to be imaged is placed in a magnetic field gradient and depending upon the frequency of protons, spatial information of the structure under is obtained. In MRS, proton nuclei and a variety of magnetic nuclei present at concentrations of 2-10mM are used to obtain information about tissue biochemistry. Precise localization to small volumes of body tissue are achieved using surface/volume coils in combination with single volume or multiple volume spectroscopic techniques (Alger 1994). Accordingly, MRS can quantify smaller concentrations of muscle tissue (example lipid) that might not be feasible with imaging techniques (Schick, Machann et al. 2002).

3.5.2 Nuclei Studied with MRS

Hydrogen, phosphorus and carbon are widely used in MRS because these nuclei produce strong MR signal and are of biomedical interest. Hydrogen is used to study hydrogen compounds such as lipids. Phosphorus is used for phosphate compounds such as ATP and PCr that play a key role in the bioenergetics of resting and exercising muscle states. Carbon is especially used for studying the metabolic fate of glycogen and metabolites of the tricarboxylic acid, which play a major role in carbohydrate metabolism (Boesch 1999). For detection by MRS, the nuclei need to be in adequate concentrations as well as fairly mobile. For example, phosphorus nuclei at 37^0C in a 1.5T magnet have 1,000,026 nuclei in the low energy state and 1,000, 000 nuclei in the

high-energy state. The MR signal therefore comes from 0.0001% of the total nuclei (Garlick and Maisey 1992). In most experiments, a threshold concentration of 0.5mmol of metabolite per kg of wet weight is required for detection with MRS (Heerschap, Houtman et al. 1999). Metabolites present in a concentration lower than milli molars (mM) are not detectable by MRS. Also, molecules that are in a bound state are not detectable. For example: ADP is a phosphate compound not detectable by ^{31}P-MRS because most of the ADP exists in the bound form.

3.5.3 Spectral Components of MRS

Identifying different nuclei in the form of different peak resonances is an outstanding feature of MRS. Each spectral peak is defined by a specific resonance frequency, height and width. **The height** of the spectral peak or the area under it yields relative measurements of metabolite concentrations. The area under a fully relaxed spectral peak is directly proportional to the concentration of nuclei that make up the peak. **The spectral width** at half its height is called the line width and gives relaxation time information because it is proportional to $1/\pi T2$ (Garlick and Maisey 1992). Spectral width is influenced by magnetic field inhomogeneties. **The position of the spectral peak** with a specific resonant frequency on the plot is expressed as parts per million. Therefore, the peaks have a specific positional relation with respect to each other in the spectra (see below for detailed description of chemical shift). Thus, the chemical shift difference between fat and water peaks will always remain 3.5 ppm in all strength fields. Accordingly, metabolites can be identified from the position of spectral peaks. Figure 3-10 shows a typical proton spectrum with its principle components.

3.5.4 Chemical Shift in MRS

The distinct peaks seen in spectroscopy can be attributed to the inherent “chemical shift” of nuclei that are tested. Even in a perfectly homogenous magnetic field, not all protons resonate at the same frequency. Depending upon the chemical environment of the nucleus in different

biochemical compounds, the same nuclei are subject to very small variations in frequency. For example: hydrogen protons in water and fat have different chemical environments and therefore their precessional/resonant frequencies are also different. In fact, protons at different sites within the same complex molecule will have different precessional frequencies. Precessional frequency of phosphorus nuclei (^{31}P) at 1T is approximately 17.18 MHz and that of hydrogen nucleus at 1T is approx 42.6MHz.

As seen in Figure 3-10, the two resonance signals of fat and water peaks are distinct. In different magnetic field strengths, these resonance frequencies will vary and therefore the numerical locator representing the two peaks will change. Therefore, in order to characterize and specify the location of MR signal irrespective of magnetic field strength, an alternative method is necessary. One method of solving this problem is to report the location of the MR signal in a spectrum relative to a reference signal. **Tetramethylsilane**, $(CH_3)_4Si$, usually referred to as **TMS**, has become the reference compound of choice for proton and carbon MR[1]. Furthermore, to correct these frequency differences for their field dependence, they are divided by the spectrometer frequency (example: 63.9 MHz for protons in a 1.5 T magnet). This difference in the resonance frequencies from a reference frequency is called chemical shift (δ). The resulting number would be very small, since Hz is divided by MHz, and is measured in parts per million (ppm), which is a dimensionless quantity independent of field strength. Chemical shift equation is given by:

$$\text{Chemical shift } (\delta) = \frac{10^6 * [\text{Frequency}_{(\text{sample})} - \text{Frequency}_{(\text{reference})}]}{\text{Frequency}_{(\text{operating})}} \quad (3\text{-}1)$$

[1] http://www.cem.msu.edu/~reusch/VirtualText/Spectrpy/nmr/nmr1.htm

Note that frequency $_{(sample)}$ is the resonant frequency of the sample signal (example fat), frequency $_{(reference)}$ is the frequency of the reference signal (TMS) and frequency $_{(operating)}$ is the frequency of the spectrometer (magnet). When chemical shift between tissues is expressed in absolute frequency, it is proportional to the magnetic field (Ray H Hashemi 1997).

3.5.5 Correction of Saturation Effects

Absolute or relative quantification of metabolites using MRS is best obtained after correction of T1 and T2 saturation effects. This is because spectroscopic sequences employed in obtaining metabolite concentrations from spectral peaks typically use relatively longer echo times and shorter repetition times (less than 6T1). While the long TE permits T2 decay and phase modulation; the shorter TR times allow for incomplete saturation of longitudinal magnetization. Collectively, there is a net loss of the acquired MRS echoes that in turn underestimate metabolite concentration. In addition, because of different T1 and T2 relaxation times of metabolites under study, simple metabolite ratios at any one given TE and TR are inaccurate representations of relative metabolite proportions. Therefore, signal correction is achieved by calculation of precise values of relaxation times and calculating for saturation factors for each metabolite. Final metabolite concentrations are obtained after correction with saturation factors.

3.5.6 Proton Spectroscopy (^{1}H-MRS)

Hydrogen protons have been used traditionally for spectroscopy because of their natural abundance in organic structures and high nuclear magnetic sensitivity. ^{1}H-MRS has been a sensitive and precise tool to quantify gross tissue fat content and derangements in lipid metabolism. Figure 3-10 represents components of a typical proton spectroscopy spectrum from a human skeletal muscle. Muscle creatine contains contributions from several metabolites including creatine phosphate. It serves as a reserve for high energy phosphates and a buffer for ATP/ADP reservoir (Castillo, Kwock et al. 1996). Interestingly, the lipid peak has contributions

from a variety of lipid components and these components can be resolved in a spectrum. Decomposition of the fat signal into components (Figure 3-11) gives specific information regarding intramyocellular lipids (IMCL) and extramyocellular lipids (EMCL). The IMCL is suggested to correspond to lipids within the myocytes that serve as an important source of energy supply during long duration endurance activities. The EMCL corresponds to the extramyocellular lipid pool that constitutes the long term storage depot for lipids (Szczepaniak, Babcock et al. 1999).

Intense signals from tissue water and fat swamps the less conspicuous signals from other metabolites of biomedical interest (muscle metabolites such as creatine, choline, etc) that are present in much lower concentrations. Successful signals from these metabolites can be obtained by using water and fat suppression techniques during acquisition of spectra or by mathematical elimination of the water peaks from the FIDs during post-processing of raw data (Hope and Moorcraft 1991).

3.5.7 Phosphorus Spectroscopy (^{31}P-MRS)

Magnetic properties of the phosphorus nucleus have gained momentum because of the pivotal role of phosphorus containing compounds in energy metabolism of skeletal muscle. Hoult et al published the first observations of metabolites from isolated rat skeletal muscle using ^{31}P-MRS in 1974 (Hoult, Busby et al. 1974). Since then, ^{31}P-MRS has made considerable impact in studying the bioenergetics of normal and pathological neuromuscular tissues and used expansively to quantify metabolic costs of various physiological processes in skeletal muscle. Almost all muscle metabolic processes involve phosphates and visualizing energy metabolism of skeletal muscle with ^{31}P-MRS (both at rest and during exercise) has proven to be valuable. One of the most outstanding features of ^{31}P-MRS is its ability to continuously obtain time dependent metabolic information from living tissues. The time resolution of ^{31}P-MRS is around 1ms and

this makes it possible to evaluate and quantify muscle oxidative and glycogenolytic energetics in-vivo (Argov and Arnold 2000).

In general, seven peaks can be identified in a phosphorus spectrum acquired from a skeletal muscle (Figure 3-12). The major peaks correspond to inorganic phosphate (Pi), phosphocreatine (PCr), and three phosphate groups of adenosine triphosphate (ATP). In addition, peaks of phosphomonosterase (PME) and phosphodiesterase (PDE) can be identified with greater spectral resolution.

Metabolite concentrations are commonly quantified from relative spectral amplitudes using ATP peaks as an internal standard. Normal Pi concentration is 3-5mM. Pi/PCr ratio is closely related to the phosphorylation potential and reflects the energy state of the muscle (Veech, Lawson et al. 1979; Chance 1984). At rest, PCr/Pi ratios range from 6-12 in healthy human muscles, depending upon the muscle studied. The single Pi peak is actually a combined peak of two molecules (HPO_4^{2-} and $H_2PO_4^-$) that are in fast exchange with each other. These acidic and basic molecules resonate at two different positions in the spectrum. Position of the Pi peak depends upon the relative concentrations of the two molecules and based on frequency shifts of the Pi peak, intracellular pH can be determined. Intracellular pH calculated based on the chemical shift difference between PCr and Pi (d) is rather accurate (up to 0.02pH units) and calculated by the following equation:

$$\text{Intracellular pH} = 6.75 + \log\left[(3.27-d)/(d-5.69)\right] \qquad (3\text{-}2)$$

This equation is derived from the more general Henderson-Hesselbach equation that defines the pH value of a sample via a titration curve (pH as a function of resonance shift);

$$\text{Intracellular pH} = \text{pKa} + \log\frac{\text{base concentration}}{\text{acid concentration}} \qquad (3\text{-}3)$$

Where, pKa is the dissociation constant of Pi (6.9), generally defined as the ability of an acid to give away its H^+ protons. Lastly, it is noteworthy that otherwise undetectable metabolites including adenosinediphosphate (ADP) and ionic concentration such as Magnesium (Mg^{+2}) can also be indirectly estimated using ^{31}P-MRS based on measurements of creatine, phosphocreatine, pH and the equilibrium constants.

As discussed below, variations in phosphate metabolite concentrations from rest to exercise and during recovery have been used to illustrate muscle energetics *in-vivo*.

3.6 Application of Magnetic Resonance Spectroscopy in Skeletal Muscle

Depending upon the nucleus studied, MRS allows observation of high-energy phosphates (^{31}P-MRS), glycogen (^{13}C-MRS) or intramyocellular fat (^{1}H-MRS). In 1955, skeletal muscle was the first biological tissue that was studied using MRS (Odeblad and Lindstrom 1955). While initial application of the technique was restricted to cell cultures and biological systems *in situ*, MRS is now widely used for a range of *in-vivo* metabolite measurements in animal and human muscle. These include a) studying different sites of the same muscle and a number of muscles simultaneously, b) yielding metabolite data from deep muscles that are difficult to access by biopsy, c) directly quantifying muscle metabolites and scrutinizing cellular components of mitochondrial metabolism instead of relying on global and/or invasive measures of oxidative phosphorylation (for example VO2max measures) d) determining real time *in-vivo* biological processes with a temporal resolution on the order of seconds, e) obtaining longitudinal measurements of skeletal muscle bioenergetics thereby permitting the study of disease progression over time and reliably quantifying effects of therapeutic interventions.

3.6.1 Role of ^{1}H-MRS : Quantify Intramuscular Fat

^{1}H-MRS has the unique ability to measure intramuscular lipid content free from muscle tissue, thereby overcoming limitations of partial volume filling (seen with MRI). In addition to the total lipid content, ^{1}H-MRS can separately quantify lipid muscle tissue into its intracellular and extra cellular components. Specifically, ^{1}H-MRS is the only currently available non-invasive tool to quantify intramyocellular (IMCL) and extramyocellular lipid (EMCL) within skeletal muscle. As discussed in chapter 1, intramyocellular lipid (IMCL) content has the potential to serve as a non-invasive marker of insulin resistance, especially in sedentary individuals. Non-invasive measures of IMCL via H-MRS are found to correlate well with IMCL quantified by electron microscopy and histochemistry using Oil Red O staining (Hinderling VB 2006). Sensitivity of ^{1}H-MRS to changes in lipid measurements is high and ^{1}H-MRS has proven to be a more valid technique for measuring IMCL than morphometry and histochemical analysis of IMCL (Schrauwen-Hinderling, Hesselink et al. 2006). The accuracy and sensitivity of ^{1}H-MRS in measuring IMCL concentrations is also sufficient to measure changes in IMCL (depletion and recovery) after exercise (Boesch, Decombaz et al. 1999). As a result, a variety of studies have utilized spectroscopy to quantify whole muscle fat and its components and their possible association with insulin resistance (Schick, Eismann et al. 1993; Machann, Haring et al. 2004; Boesch 2007).

3.6.2 Role of ^{1}H-MRS: Assess Muscle T2 Characteristics

Chemical shift differences between protons bound to muscle and protons bound to fat yield separate water and lipid peaks; thereby allowing measurements of T2 relaxation properties of muscle and fat tissues separately. Accordingly, muscle tissue composition including muscle damage and edema is more reliably measured using ^{1}H-MRS measures. Investigators are using this ability of ^{1}H-MRS to decipher between fatty and damaged muscle (Walter, Cordier et al.

2005) and to identify muscle degeneration in a variety of muscle disorders (Bongers, Schick et al. 1992).

3.6.3 Role of ^{31}P-MRS: Quantify Resting Muscle Metabolites

At rest, most of the muscle energy supplies come from the formation of ATP from ADP and inorganic phosphate (Pi) that takes place in the electron transport chain of the mitochondrion (mitochondrial oxidative phosphorylation). Metabolism at rest occurs at a low rate. Accordingly, it is easy to obtain baseline ^{31}P-MRS spectrum. Studies report ^{31}P-MRS measured PCr/Pi ratios as reliable markers of phosphorylation potential (Chance 1984; McCully, Kent et al. 1988). Phosphorylation potential is the energetic potential of the cell. The variables [ADP][Pi]/[ATP] describe the effects of energy demand and potential for ATP supply by oxidative phosphorylation. Alterations in resting metabolite content, metabolite ratios and change in pH reflect disturbances in the metabolic pathways and/or structural dysfunctions of mitochondria or a diseased state of the muscle as a whole (Heerschap, Houtman et al. 1999; Mattei, Bendahan et al. 2004). Increase in resting Pi content and Pi/PCr ratios of skeletal muscle obtained by ^{31}P-MRS have been observed in a variety of disorders including primary mitochondrial diseases, myopathies, muscle injury, disuse and denervation (McCully, Kent et al. 1988; Argov and Arnold 2000; Tartaglia, Chen et al. 2000). In the lower limb muscle disuse model, Pathare et al have reported an increase in basal Pi along with concurrent decrease in the PCr/Pi ratios in the plantarflexor muscles of individuals following cast immobilization secondary to ankle fracture (Pathare, Walter et al. 2005; Pathare, Vandenborne et al. 2007). Moreover, the elevated Pi content and Pi/PCr ratios were found to significantly impact the force generating capacity of the skeletal muscle. Elevated Pi/PCr ratios are also demonstrated during muscle injury (McCully, Kent et al. 1988; Pathare, Vandenborne et al. 2007). Furthermore, right shift of the Pi peak

relative to the PCr peak in a ^{31}P spectrum implies elevated H+ ions produced by lactate and hence an acidic state of the muscle.

Information provided by resting spectra reflects metabolic state of the muscle at rest. Abnormalities in muscle bioenergetics however, are better characterized in dynamic experiments performing ^{31}P-MRS during exercise and recovery. The subsequent section describes one of the most popular applications of ^{31}P-MRS - measurement of metabolic oxidative capacity of the skeletal muscle.

3.6.4 Role of ^{31}P-MRS: Identify Fiber-type and Muscle Fatigue

Three individual Pi peaks have been identified in the ^{31}P-MRS spectrum of the human gastrocnemius muscles following brief periods of muscle contraction (Vandenborne, McCully et al. 1991). Based upon distinct positions of the three peaks in the MRS spectrum that correspond to heterogeneity in pH, the authors hypothesized that the three Pi spectral peaks correspond to slow oxidative, fast twitch oxidative and fast twitch glycolytic fibers. Similarly, split in the Pi peak has been reported by Kutsuzawa et al in patients with chronic respiratory impairment during moderate exercise intensity of the forearm muscles; implying the contribution of different fiber types to muscle work. Interpretation of muscle fiber type is also based upon the ratios of phosphate metabolites (Kutsuzawa, Shioya et al. 1992). Resting Pi/PCr ratios are higher in red muscle as compared to white muscles because of baseline higher levels of Pi in red muscle and higher PCr levels in white muscle (Meyer, Brown et al. 1985; Kushmerick, Moerland et al. 1992).

Amongst various other sources of peripheral muscle fatigue, altered levels of muscle metabolites (example excessive Pi levels, H^+ and $H2PO4^-$) are purported as a potential source of skeletal muscle fatigue at the myocyte level (Kevin K McCully 1994). Fatigue is described as the decreased force generating capacity of the muscle. Metabolic aspects of muscle fatigue can be

evaluated by monitoring concentration of oxidative metabolites and pH of exercising muscle. For example: A strong relationship has been observed in the time frame of fatigue in the dorsiflexor muscles of healthy subjects and the accumulation of $H_2PO_4^-$ (Kent-Braun, Ng et al. 2002).

3.6.5 Role of ^{31}P-MRS: Measure Muscle Oxidative Capacity

One of the most valuable contributions of ^{31}P-MRS is the non-invasive measurement of the oxidative capacity of skeletal muscle (McCully, Fielding et al. 1993; Kemp, Sanderson et al. 1996; Argov and Arnold 2000). Measuring oxidative capacity via ^{31}P-MRS has gained tremendous attention because of participation of energy rich phosphates in muscle bioenergetics. One of the most reliable measures of oxidative capacity using ^{31}P-MRS is the rate of PCr resynthesis. PCr recovery rates have been extensively used in both healthy and diseased muscles as estimates of muscle oxidative capacity (Meyer 1988; Paganini, Foley et al. 1997; McCully, Mancini et al. 1999; Argov and Arnold 2000; Kent-Braun and Ng 2000; Pathare, Vandenborne et al. 2007).

Energy released from ATP hydrolysis forms the principal energy source for muscle metabolism during rest and work. The immediate source of ATP at onset of exercise is provided by hydrolysis of PCr catalyzed by the enzyme creatine kinase.

$$MgADP^- + PCr + H^+ \xleftrightarrow[\text{Creatine Kinase}]{} MgATP^{2-} + \text{Creatine} \qquad (3\text{-}4)$$

The resultant ATP is quickly hydrolyzed into inorganic phosphate (Foley, Jayaraman et al.) and ADP while releasing energy enough to meet energy demands of the cell.

$$PCr + H^+ \longleftrightarrow \text{Creatine} + \text{Inorganic phosphate} \qquad (3\text{-}5)$$

Noteworthy, homeostatic mechanisms exist within the myocyte that couple overall ATP utilization with ATP synthesis; thereby maintaining nearly steady concentrations of ATP (Erecinska and Wilson 1982; Kushmerick 1995). In this respect, one of the most important

contributions is the *temporal* and *spatial* buffer role of creatine kinase reaction in maintaining energy homeostasis. High PCr content and creatine kinase enzyme activity in skeletal muscle makes the creatine kinase equilibrium reaction an extremely efficient enzymatic system that is capable of buffering transient changes in ATP (Kushmerick and Meyer 1985). A closer look at bioenergetics' data during moderate exercise reveals that while ATP is held constant, the PCr levels continue to deplete (PCr hydrolysis). The following steps (Bessman and Geiger 1981) briefly describe the creatine kinase buffer phenomena (Figure 13) : a) During beginning of exercise, cystolic PCr hydrolysis produces ATP b) Subsequent cystolic respiration (aerobic and anaerobic) is controlled through kinetic control of ADP (respiratory control of ADP) (Dudley, Tullson et al. 1987) while the reverse creatine kinase continues to maintain ATP levels (*temporal buffer* role of the creatine kinase reaction by myofibrillar creatine kinase) c) the creatine-kinase reaction at sites of ATPase activity also triggers the creatine-phosphocreatine shuttle mechanism such that creatine released from sites of contraction diffuses (shuttled) into the mitochondria d) ATP produced by oxidative phosphorylation and glycogenolysis is used by mitochondrial creatine kinase isoenzyme to resynthesize PCr. This PCr is then shuttled back for participation in the CK reaction to release ATP at the sites of muscle contraction. Thus, the mitochondrial creatine kinase serves as an intermediate in the transfer of high energy phosphate from sites of ATP production (mitochondria and glycolytic loci) to ATP consuming locations in the myocyte (Kushmerick 1995; Heerschap, Houtman et al. 1999). Therefore, while muscle creatine kinase controls the backward reaction ($PCr + ADP + H^+ \rightarrow$ Creatine + ATP) outside the mitochondria; the forward reaction is controlled by mitochondrial creatine kinase isoenzyme (Sahlin, Harris et al. 1979). In this regard, the creatine kinase reaction acts as a *spatial buffer* and maintains cell ATP homeostasis (Figure 3-13) e) However, the rate of ATP release from oxidative

phosphorylation and glycogenolysis is not as fast as the rate of ATPase reaction' thereby accounting for depletion of PCr; which is in proportion to ATP turnover until the end of the workloads. Large declines in PCr are therefore accompanied by small or no changes in ATP and ADP. At steady workloads, the oxidative phosphorylation reaches a steady state of ATP release, thereby leveling off PCr levels as long as no further demand for ATP arises. [Note that the shuttle mechanism explained above is only one theory that describes PCr replenishment by ATP in the skeletal muscle. This metabolite homeostasis can also occur by simple diffusion processes (the buffer and spatial roles); which are the alternate and more modern explanations to describe this homeostatic mechanism (Kushmerick 1995, Kushmerick and Meyer 1985)].

3.6.6 Relationship between PCr Recovery Rates and Muscle Oxidative Capacity

During recovery from exercise, ATP breakdown is minimal and PCr levels return to baseline values. Studies have well established that during recovery from exercise, glycolysis ceases and PCr in the muscle cell is replenished at the expense of ATP produced via mitochondrial oxidative phosphorylation (Taylor, Bore et al. 1983; Meyer 1988; Quistorff, Johansen et al. 1993; Kemp, Roberts et al. 2001; Mattei, Bendahan et al. 2004). Indeed, for steady state, low intensity exercise, where no change in intracellular pH (Phillips, Wiseman et al.) is expected, a constant positive relationship exists in the time scales and rates of oxygen consumption in the mitochondria following exercise and PCr recovery rates (Piiper and Spiller 1970; Meyer 1988; McCully, Iotti et al. 1994; Thompson, Kemp et al. 1995). These results confer that PCr resynthesis after exercise is mediated via oxidative phosphorylation. Accordingly, rate constant of PCr recovery (PCr recovery rates) is conventionally been recognized as an index of mitochondrial oxidative capacity.

PCr resynthesis follows a pseudo first order kinetics and the rate constant is described by a monoexponential curve as long as PCr is depleted by a sufficient amount (~50%) and pH does

not decline below 6.75 units (Taylor, Bore et al. 1983; McCully, Iotti et al. 1994; Boesch 2007). In addition, for the rate constants of PCr recovery to correctly reflect oxidative capacity, several assumptions are made: a) the creatine kinase reaction is in equilibrium b) presence of an intact vascular (hence oxygen) supply to the muscle during recovery c) the PCr resynthesis is predominantly due to ATP released by oxidative processes (thus anaerobic ATP production is negligible) (Meyer 1988; Paganini, Foley et al. 1997). Furthermore, PCr resynthesis following depletion necessitates the presence of intact blood supply and oxygen availability (Sahlin, Harris et al. 1979; Quistorff, Johansen et al. 1993).

Importantly, while PC recovery rates can be used as indices of oxidative capacity, absolute estimations of metabolic capacity from PCr recovery data is possible using rigorous theoretical framework (Paganini, Foley et al. 1997). For practical purposes, the rate constant of PCr recovery (k) that reflects muscle oxidative capacity is calculated using the following equation (Hartkopp, Harridge et al. 2003; Kitahara, Hamaoka et al. 2003):

$$PCr\ (t) = PCr_0 + \Delta PCr\ (1\text{-}e^{-kt}) \qquad (3\text{-}6)$$

Where, PCr is the concentration of PCr at a given time t during post exercise recovery; PCr_0 is the PCr concentration at end of exercise and ΔPCr is the change in PCr concentration after recovery from exercise. Note that the rate constant (k) is influenced by change in pH such that lower pH decreases the rate constants of PCr recovery (Bendahan, Confort-Gouny et al. 1990; McCully, Iotti et al. 1994; Paganini, Foley et al. 1997; Heerschap, Houtman et al. 1999). Alterations in pH apparently alter the equilibrium state of the creatine kinase reaction and/or mitochondrial ATP yield (Bendahan, Confort-Gouny et al. 1990; McCully, Iotti et al. 1994). Accordingly, measurements of oxidative capacity typically accompanying pH decrease do not correctly reflect oxidative capacity. The inverse of the rate constant kPCr is called the time

constant of PCr recovery (τ PCr,) and is related to peak oxygen uptake (VO_2) and maximal oxygen consumption (VO_2 max) (Thompson, Kemp et al. 1995). The τ PCr is reported to be independent of exercise intensity and end exercise levels of PCr.

Another reliable measure, initial post exercise PCr resynthesis rate (Vmeas), is also demonstrated to reflect mitochondrial oxidative capacity and is less affected by the end exercise PCr levels and pH (Meyer 1988; Kemp, Thompson et al. 1994; Lodi, Kemp et al. 1997). This initial rate of PCr resynthesis during the first few seconds is estimated from the first two-three data points in recovery and is quantified by:

$$Vmeas = (d[PCr]/dt) \quad (3\text{-}7)$$

Also, a more complete estimation of maximum mitochondrial capacity (Qmax) has also been used (Kemp, Thompson et al. 1994);

$$Qmax = k\,[basal\ PCr] \quad (3\text{-}8)$$

where k is the rate constant of PCr recovery and Δ [PCr] is the basal PCr levels.

As mentioned above, increase in ADP levels that follow exercise, tend to return to baseline levels soon after exercise. Faster the recovery of ADP levels, faster is ATP formed and therefore this implies an increased efficiency of mitochondria. Indeed, ADP concentrations in myocytes are known to regulate mitochondrial ATP synthesis. As ATP is used up during exercise, the [ADP] increases and return of [ADP] levels back to normal are indicative of in vivo mitochondrial function or oxidative capacity (Argov, De Stefano et al. 1996). Few studies have in fact proven that the rate of decline in ADP is a more sensitive measure of oxidative capacity than PCr recovery rates because of its robustness to changes in intracellular pH (Kemp and Radda 1994; Argov and Arnold 2000). However, since ADP levels are not directly measurable, it is less commonly used. Also, owing to its relatively lower concentration; [ADP] cannot be

directly measured from a normal ^{31}P spectrum; but can be estimated using ^{31}P-MRS assuming a constant magnesium ion concentration (Kemp, Sanderson et al. 1996; Durozard, Gabrielle et al. 2000).

3.7 Summary

This chapter reviews the fundamental concepts of MR with main focus on MRI and MRS applications in skeletal muscle. MR has become the investigative tool of choice because of its non-invasiveness, ability to perform longitudinal studies and obtain a wealth of anatomical and biochemical information of skeletal muscle. While MRI more clearly assesses muscle morphology, MRS estimates physiological metabolic processes in real time. Various applications of MRI in skeletal muscle include assessment of muscle size, identification of muscle damage and quantification of fatty tissue infiltration. The most common nuclei used in MRS include proton and phosphorus. Through its unique ability to decipher between protons in water and fat peak in muscles, ^{1}H-MRS has gained widespread application in characterizing tissue T2 relaxation properties and separately quantifying IMCL and EMCL fat components. ^{31}P-MRS has been utilized to estimate muscle pH and quantifying energy rich phosphates that participate in basic physiological processes. The high time resolution and the ability to quantify metabolites in real time has made ^{31}P-MRS a unique non-invasive tool to reliably measure muscle oxidative capacity *in-vivo*.

Table 3-1. Longitudinal (T1) relaxation times (in milliseconds) of water and lipid components obtained from **human** skeletal muscle at different magnetic field strengths.

Reference	Muscle	Magnetic field strength	Water	Fat	IMCL	EMCL
Bruhn 1991	Plantar flexors	1.5T	n/a	300	n/a	n/a
Bongers 1992	Plantar flexors	1.5T	1100-1400	280-330	n/a	n/a
Schick 1993	Soleus	1.5T	1100-1500	270-280	n/a	n/a
Sinha 2002	Soleus	2.1T	1220-1370	n/a	306-378	221-461
Hwang Jong Hee 2001	Soleus	4T	1300-2300	n/a	340-440	340-440

Table 3-2. Transverse (T2) relaxation times (in milliseconds) of water and lipid components obtained from **human** skeletal muscle at different magnetic field strengths.

Reference	Muscle	Magnetic field strength	Water	Fat	IMCL	EMCL
Bongers 1992	Plantar flexors	1.5T	n/a	75-100	n/a	n/a
Bruhn 1991	Plantar flexors	1.5T	30	90	n/a	n/a
Szczepaniak 1999	Soleus	1.5T	37-43	n/a	82-90	66-76
Schick F, 1993	Soleus	1.5T	50	70-85	n/a	n/a
Sinha R, 2002	Soleus	2.1T	29-34	n/a	70-83	76-86
Hwang Jong Hee 2001	Soleus	4T	21-31	n/a	68-82	58-78

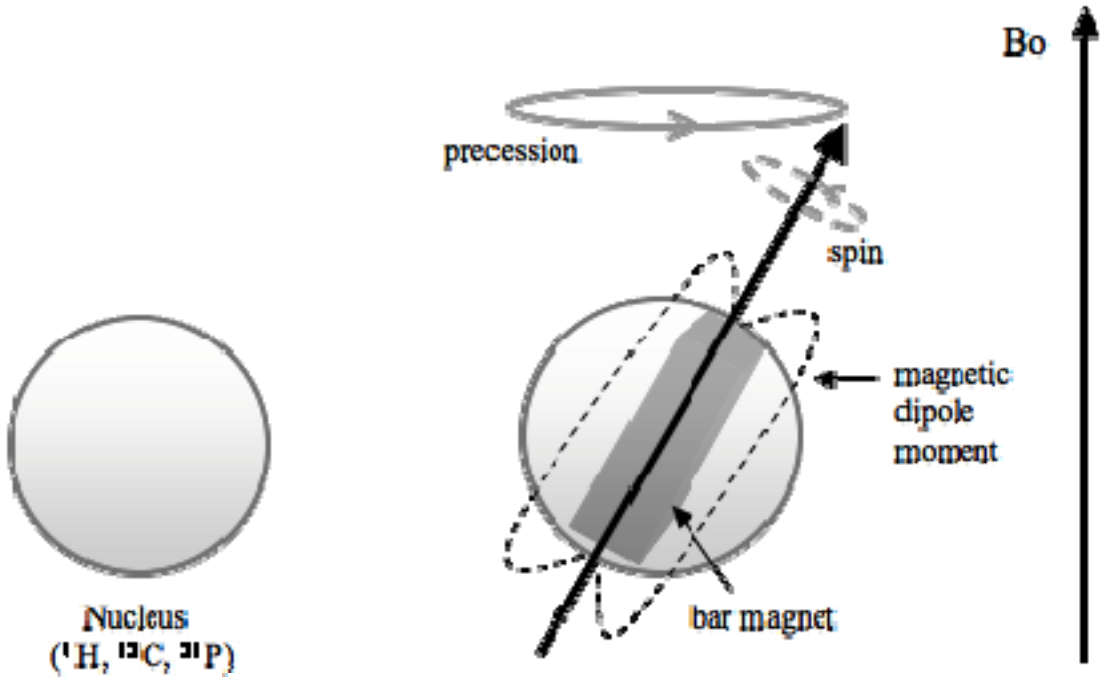

Figure 3-1. Schematic representation of a nucleus and its behavior as a bar magnet in an external magnetic field Bo

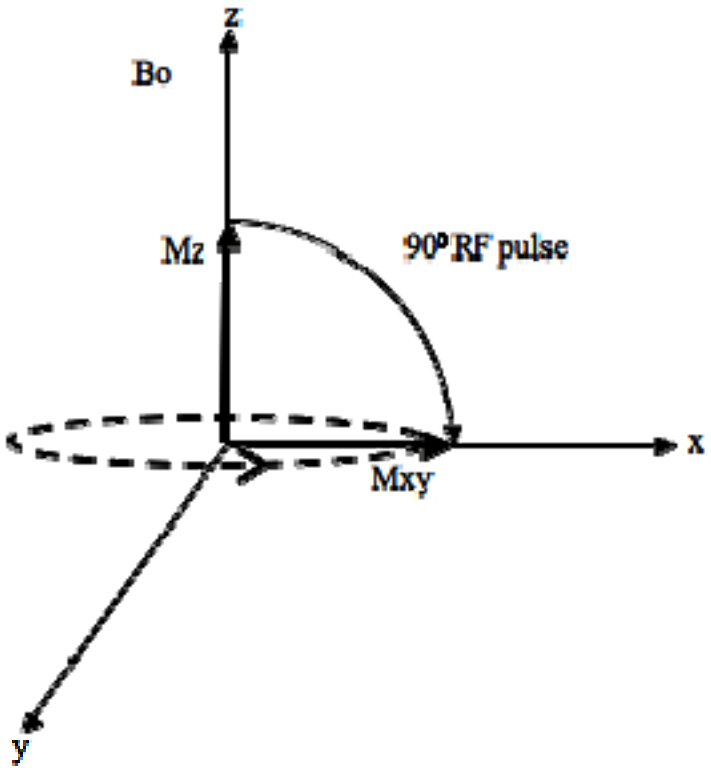

Figure 3-2. Application of a 90° RF pulse.

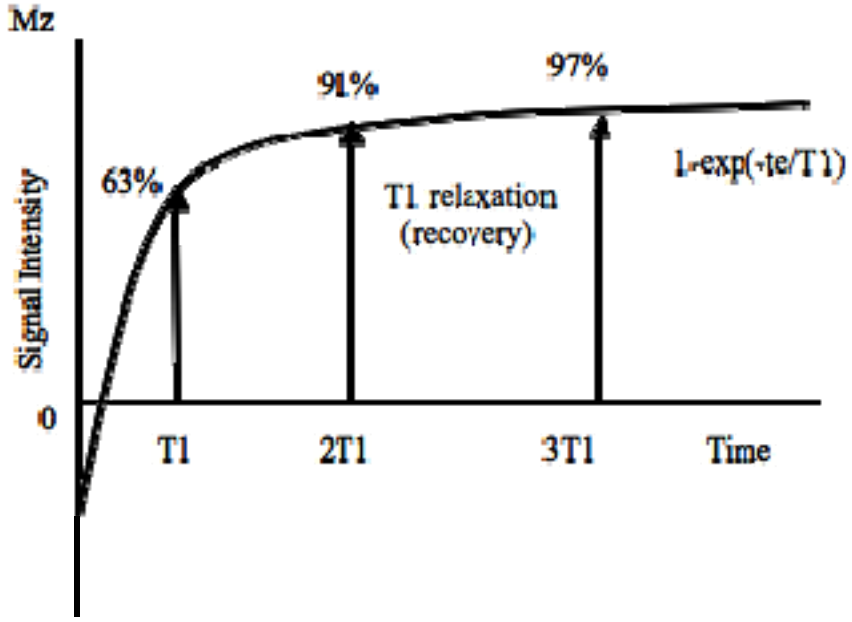

Figure 3-3. T1 relaxation time

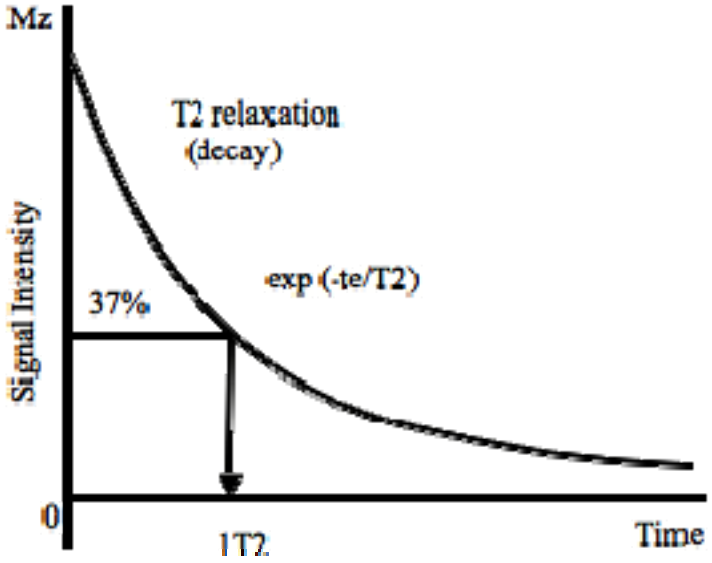

Figure 3-4. T2 relaxation time

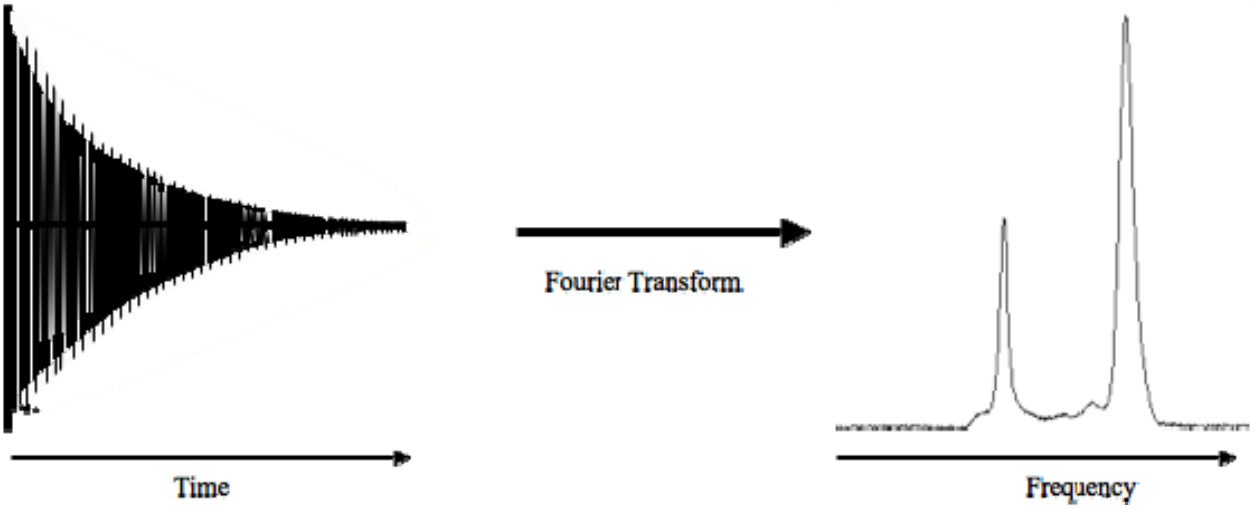

Figure 3-5. Fourier transform of the FID signal.

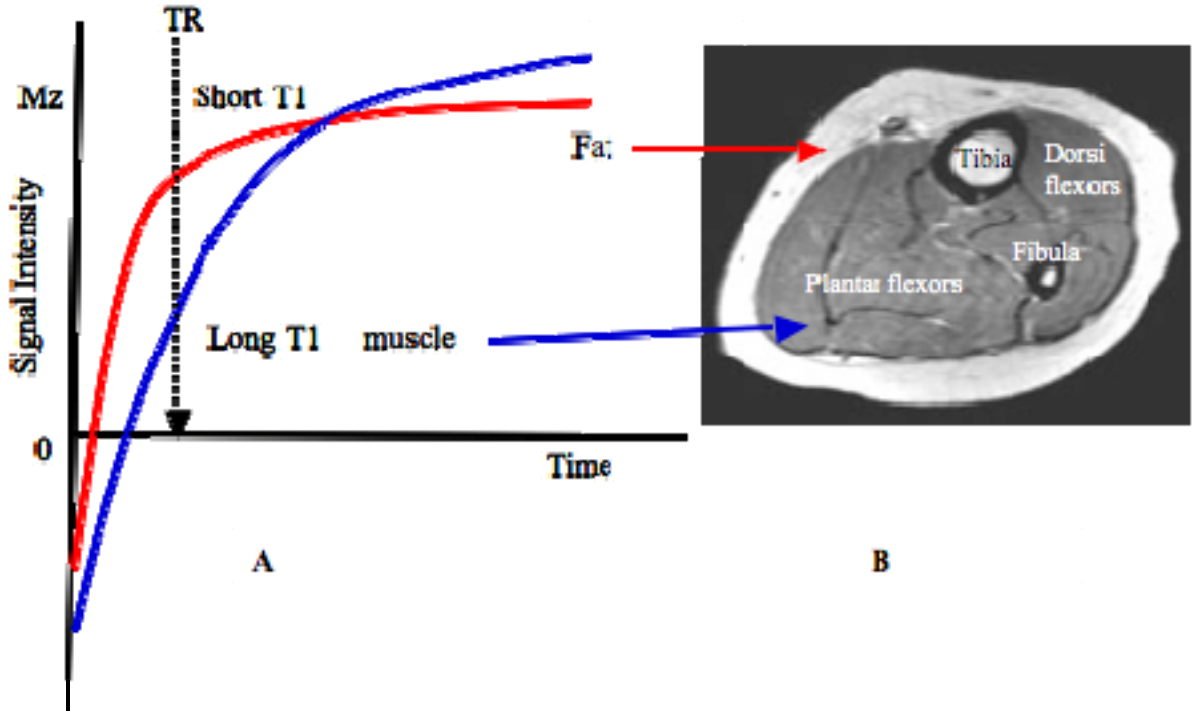

Figure 3-6. Schematic representation of two tissues with different T1 relaxation times. A) Faster T1 relaxation times of fat tissue and relatively slower T1 relaxation times of skeletal muscle tissue. B) Representative T1 weighted trans-axial image of a healthy human calf muscle obtained 3T.

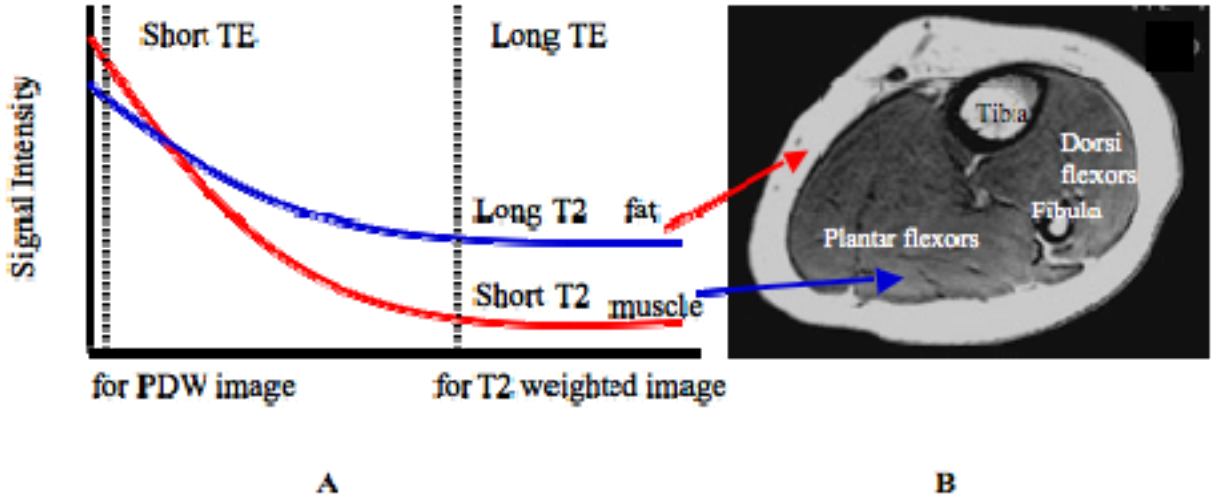

Figure 3-7. Schematic representation of two tissues with different T2 relaxation times. A) Faster T2 relaxation times of muscle tissue and relatively slower T2 relaxation times of fat tissue. B) Representative T2 weighted trans-axial image of a healthy human calf muscle obtained at 3T.

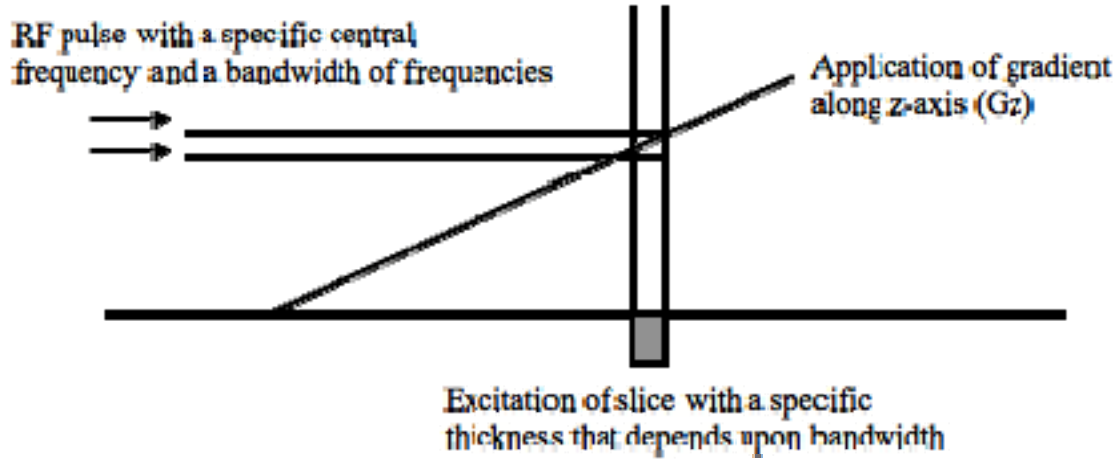

Figure 3-8. Bandwidth of frequencies excites a specific width of slice in the sample.

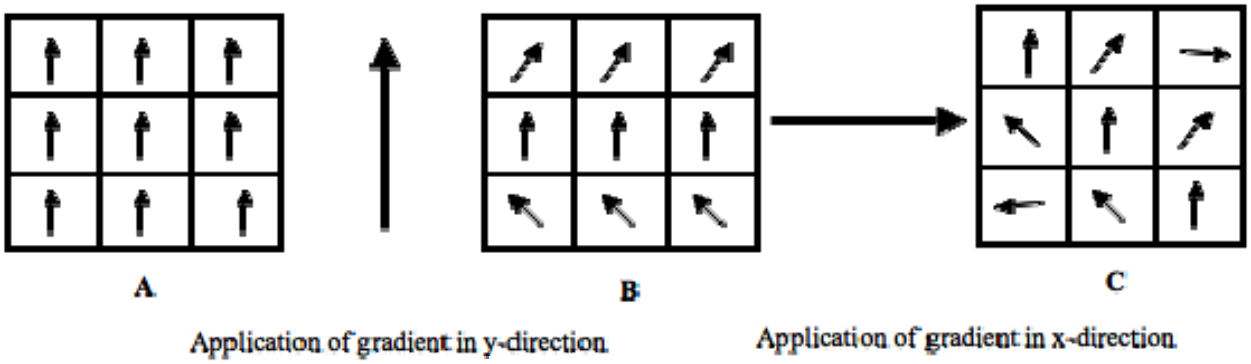

Figure 3-9. Frequency and phase encoding gradient effects on spins. A) Spins (arrows depict spin phases) in all the rows of the grid are aligned in the same direction of the main magnetic field before application of a phase encoding gradient (Gy) B) After application of Gy, spins in the upper row experience a higher magnetic field and spins in the lowest row experience the least magnetic field. C) After application of frequency encoding gradient, spins in the upper and lower rows change phases while those in the center row experience no change in magnetic field.

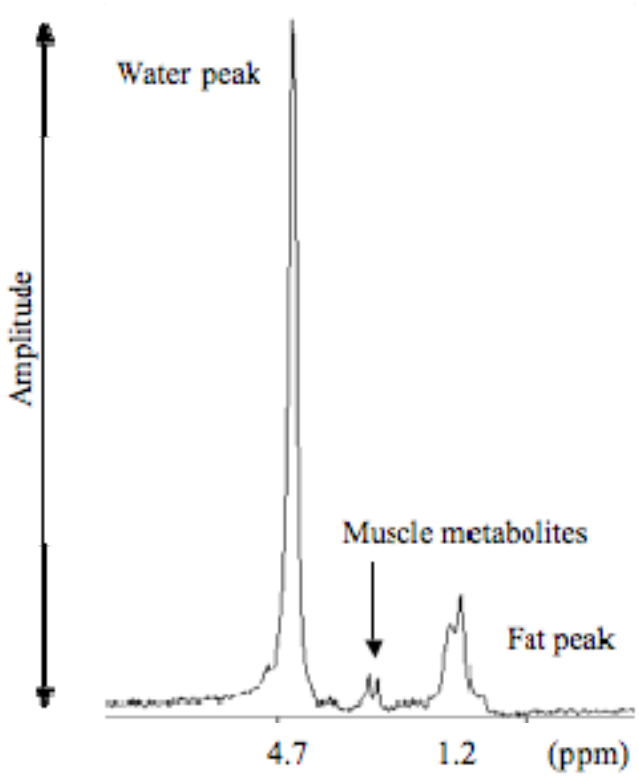

Figure 3-10. Representative ^{1}H-MR spectrum of a healthy human soleus muscle at 1.5Tesla.

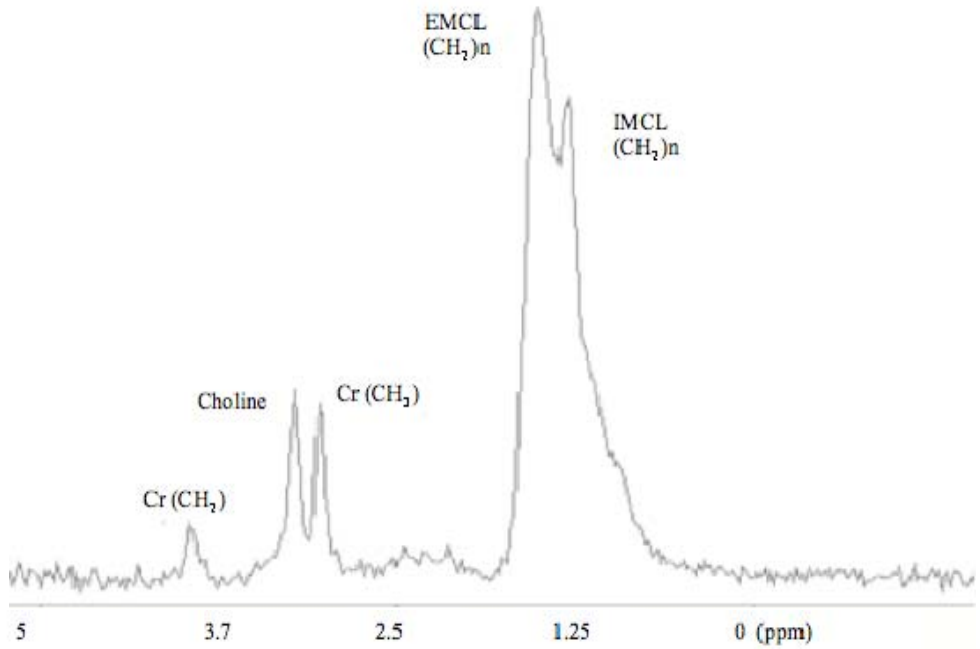

Figure 3-11. Representative ^{1}H-MR spectrum of a human skeletal muscle at 1.5Tesla showing decomposition of the lipid peak into its IMCL and EMCL components after water suppression (at ppm = 0). The creatine (Cr) and choline peaks represent muscle metabolites.

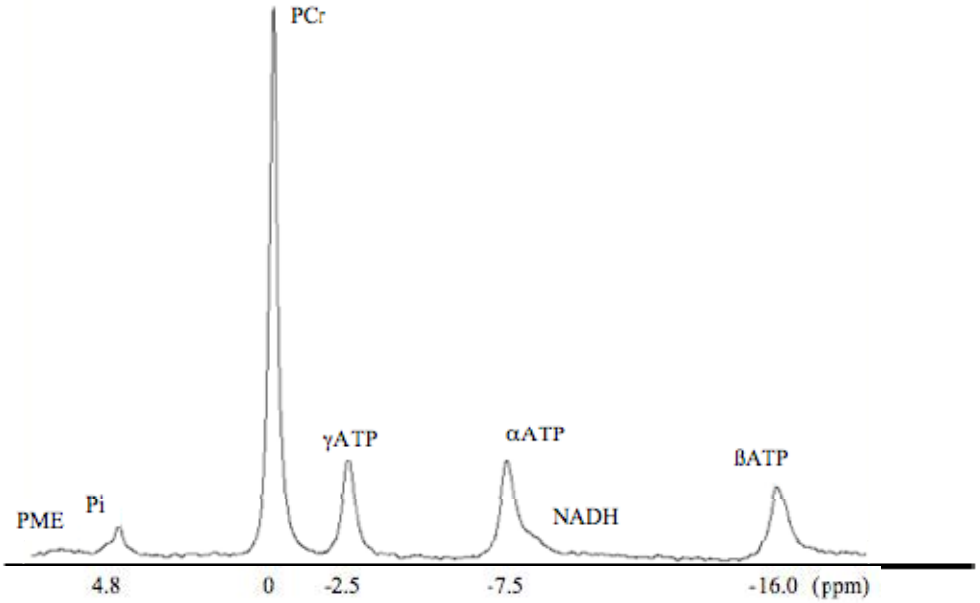

Figure 3-12. Representative typical ^{31}P-MR spectrum of a rat calf muscle at 11Tesla (NADH = nicotinamide dehydrogenase; PME= phosphomonoesterase)

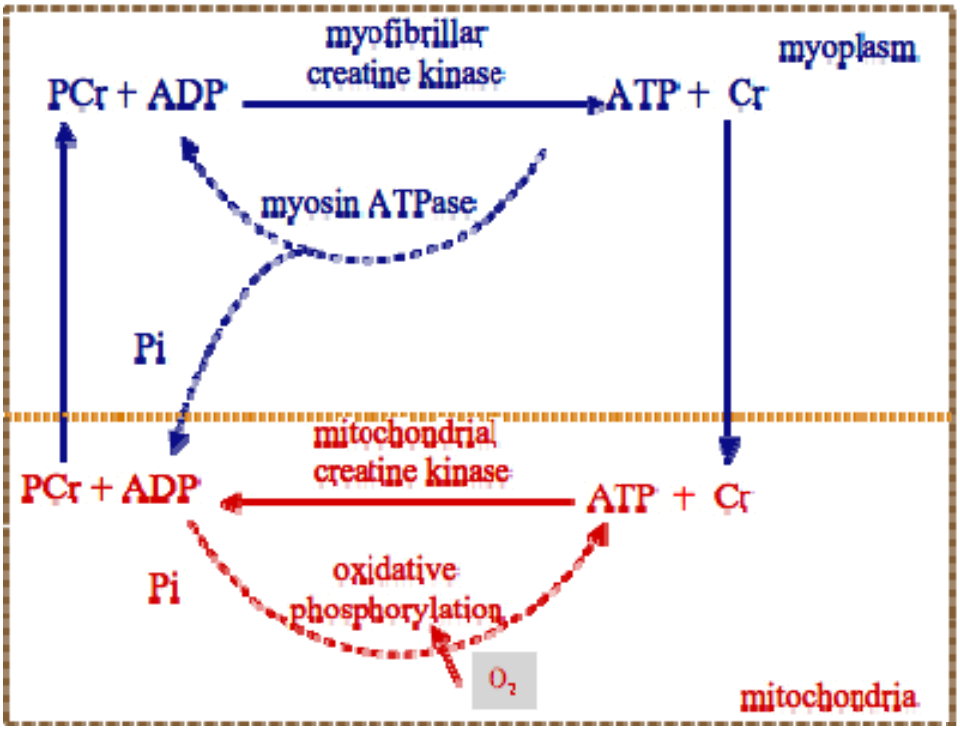

Figure 3-13. Schematic representation of the creatine-phosphocreatine shuttle and buffer role of the creatine-kinase reaction muscle.

CHAPTER 4
OUTLINE OF EXPERIMENTS

The overall objective of this dissertation was to investigate the lower extremity skeletal muscle adaptations after incomplete spinal cord injury. An outline of aims and hypotheses related to experiments is given below.

4.1 Experiment One

4.1.1 Specific aim

a) To quantify lower extremity skeletal muscle size in persons with chronic incomplete spinal cord injury (SCI) and in age-matched healthy individuals b) To determine atrophic response in anti-gravity versus the non anti-gravity muscles c) To examine the impact of ambulatory ability on muscle size.

Maximum cross sectional areas (CSA) of the thigh and calf muscles were quantified using high-resolution magnetic resonance imaging (MRI).

4.1.2 Hypotheses

a) Chronic incomplete SCI leads to a decline in lower extremity muscle CSA.

b) Greater decreases are observed in the lower extremity anti-gravity muscles as compared to the non anti-gravity muscles.

c) Persons with incomplete SCI who use a wheelchair as primary means of mobility show a larger atrophic response than persons with SCI who do not use a wheelchair for ambulation.

4.2. Experiment Two

4.2.1 Specific Aim

a) To characterize muscle characteristics via T2 relaxation times of lower extremity muscles in persons with incomplete SCI and compare that with age-matched controls. b) To

quantify the intramuscular lipid content along with intramyocellular and extramyocellular lipid composition of the soleus muscle after incomplete SCI in humans.

Skeletal muscle T2 relaxation properties and soleus muscle lipid content are determined using combinations of MRI and proton spectroscopy (^{1}H-MRS).

4.2.2 Hypotheses

a) Persons with incomplete SCI show alterations in the T2 relaxation times of the lower extremity muscles as compared to healthy individuals.

b) Persons with incomplete SCI show elevated soleus muscle lipid content along with elevations in both the intramyocellular (IMCL) and extramyocellular lipid (EMCL) components as compared to healthy individuals.

4.3 Experiment Three

4.3.1 Specific Aim

To assess the impact of two and nine weeks of locomotor training on markers of muscle damage in persons with incomplete SCI. Lower leg skeletal muscle T2 relaxation properties are determined using combinations of MRI and spectroscopy (^{1}H-MRS) measures.

4.3.2 Hypothesis

Two and nine weeks of locomotor training alter the T2 relaxation properties of lower leg muscles of persons with incomplete SCI.

4.4. Experiment Four

4.4.1 Specific Aim

a) To assess the impact of nine weeks of locomotor training on the lower extremity muscle size of persons with incomplete SCI. b) To assess the impact of nine weeks of locomotor training on the soleus muscle composition of persons with incomplete SCI.

Skeletal muscle CSA and soleus muscle lipid content are determined using combinations of MRI and spectroscopy (^{1}H-MRS).

4.4.2 Hypotheses

a) Locomotor training attenuates the atrophic response and increases the muscle cross-sectional area in lower extremity skeletal muscle size of persons with incomplete SCI.

b) Locomotor training alters the lipid content of soleus muscle in persons with incomplete SCI, with changes seen both in the IMCL and EMCL.

4.5. Experiment Five

4.5.1 Specific Aim

a) To determine the impact of acute spinal contusion (one week) on the content of resting phosphates, and hence the phosphorylation potential of rat calf muscle after spinal cord contusion. b) To longitudinally monitor alterations in the phosphorylation potential of the calf muscles.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) at a high magnetic field strength (11T) was used to monitor basal phosphate metabolites for three weeks after spinal cord contusion. Intracellular quantification of phosphate metabolites was achieved using biochemical assays.

4.5.2 Hypotheses

a) Immediately after spinal cord contusion (one week), there is an alteration in the resting muscle phosphate content; and hence a change in the phosphorylation potential of the paralyzed hind limb muscle.

b) Muscle phosphate levels approach control values by three weeks after spinal cord contusion.

4.6. Experiment Six

4.6.1 Specific Aim:

a) To determine the impact of acute spinal contusion (one week) on oxidative capacity of rat hind limb muscle after spinal cord contusion. b) To longitudinally monitor alterations in the skeletal muscle oxidative capacity of the hind limb muscles.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) was performed on the animal hind limb muscle using an electrical stimulation protocol to quantify *in-vivo* muscle bioenergetics in real time. Measurements were obtained once weekly for three weeks starting at one week post injury.

4.6.2 Hypotheses

a) Immediately after spinal cord contusion (one week), there is a decrease in the oxidative capacity of the paralyzed hind limb muscle.

b) Muscle oxidative capacity approach control values after three weeks of spinal cord contusion.

CHAPTER 5
METHODOLOGY

In this dissertation, experiments one through four are focused on studying muscle adaptations in humans with incomplete spinal cord injury. Experiments five and six are conducted in a rat model of spinal contusion injury. While non-invasive MR techniques are employed as the key tool for all experiments; combinations of biochemical assays, histology and pap smears have been employed as additional methodologies to supplement MR findings in the animal studies. The following sections discuss relevant experimental protocols and data analyses relevant to both human and animal studies.

5.1 Human Studies

5.1.1 Subjects

Persons with incomplete SCI and age-matched controls were recruited for this study. Prior to participation in the study, all subjects were informed of the study purpose and all provided written informed consent as approved by the Institutional Review Boards at the University of Florida.

Able-bodied controls: Age, weight, height and gender matched individuals from the local University of Florida setting were recruited as healthy volunteers for the study. Control subjects were recreationally active, but not engaged in any rigorous exercise program. Controls were screened for MR compatibility before participation.

Individuals with incomplete spinal cord injury: Individuals with SCI were recruited from the local community at the University of Florida in Gainesville, FL or at the University of Georgia, Athens, GA. The inclusion criteria for participants were 1) diagnosis of traumatic SCI at cervical or thoracic levels (C4-T12) resulting in upper motor neuron lesions in the lower extremity, 2) history of SCI as defined by the American Spinal Injury Association (ASIA)

Impairment Scale categories C or D, and 3) a medically stable condition at the time of testing 4) persons who were MR compatible. Participants had varied ambulatory capabilities and accordingly, used a wheelchair, cane, crutches and/or ankle foot orthosis for assistance in mobility.

5.1.2 Clinical Assessments

American Spinal cord Injury Association (ASIA) scores for neurological classification of spinal cord injury: The ASIA impairment scale for neurological classification of spinal cord injury is a nominal measure for measuring completeness of injury (ASIA 2001). Injury to the spinal cord is considered complete if there is "absence of sensory and motor functions in the lowest sacral segments" and incomplete if there is "preservation of sensory or motor function below the level of injury, including the lowest sacral segments". Impairments in muscle strength and sensory function are graded by the ASIA impairment scale (ASIA 2001) as follows:

ASIA A - Complete: No sensory or motor function is preserved in sacral segments S4-S5.

ASIA B - Incomplete: Sensory, but not motor, function is preserved below the neurological level and extends through sacral segments S4-S5.

ASIA C - Incomplete: Motor function is preserved below the neurological level, and most key muscles below the neurological level have muscle grade less than 3.

ASIA D - Incomplete: Motor function is preserved below the neurological level, and most key muscles below the neurological level have muscle grade greater than or equal to 3.

ASIA E - Normal: Sensory and motor functions are normal.

The sacral fibers are located more at the periphery of the cord making them least susceptible to injury after a spinal cord traumatic event. Accordingly, after a SCI, sacral-sparing is evidence of the physiologic continuity of spinal cord long tract fibers. "Indication of the presence of sacral fibers is of significance in defining the completeness of the injury and the

potential for some motor recovery. This finding tends to be repeated and better defined after the period of spinal shock".

Lower Extremities Motor Score (LEMS): This is a commonly used measure to define the motor capabilities after SCI. This score uses the manual muscle testing scores of the ASIA key muscles in both lower extremities with a total possible score of 50 (i.e., maximum score of 5 for each key muscle per extremity). Accordingly, a LEMS score of 20 or less indicates that individuals with incomplete SCI are less likely to be community ambulators. In contrast, a LEMS of 30 or more suggests a better likelihood for community ambulation (ASIA 2001).

Walking Index for Spinal Cord Injury (WISCI): Originally developed by the international study group, the Walking Index for Spinal Cord Injury (WISCI) score is a scale that indicates the ability to walk after SCI. It is a reliable and valid tool that has been found to correlate well with ASIA scores (Burns, Golding et al.). Researchers and clinicians have widely used the WISCI score to assess the walking ability after SCI. The testing incorporates assessing the ability to walk for 10meters with the help of assistive aids, crutch and physical assistance of one or two persons. Accordingly, the severity of walking impairment is graded from most to least severe on a scale of 0 to 20. A score of zero implies inability to stand and/or walk at all with assistance and a score of 20 implies the ability to ambulate independently for 10m (Ditunno, Ditunno et al. 2000). Note worthily, the scale is not reflective of the functional ambulatory status of the individual in the community.

5.1.3 Locomotor Training

Locomotor training (LT) consisted of 9 weeks of step training (30 minutes, 5days/week) on the treadmill with body weight support and manual assistance followed by over ground training (20 minutes). Bodyweight support, initially set to 40% of the subjects' body weight was adjusted to maintain proper limb kinetics while also maximizing bilateral limb loading. If

necessary, manual assistance was provided by trainers to assist in correct stepping. In addition, assistance was provided in maintaining an upright trunk by stabilizing the pelvis. Subjects were encouraged to swing their arms voluntarily or with the aid of poles. Verbal encouragement by trainers along with visual cues provided by a mirror placed in front of them served as additional sensory cues to facilitate a near-normal walking pattern. Speed of treadmill stepping was kept in a range consistent with normal walking, optimized for each subject (2.0-2.8 miles/hr). Training sessions were interspersed with adequate rest periods. During each rest break, the participant stood with minimal BWS required to maintain balance with minimal assistance from the trainers. Initially, treadmill sessions required up to 60 minutes to achieve 30 minutes of stepping, but the amount decreased with improved walking ability and endurance such that stepping could be completed in 45 minutes or less. Progression of training was achieved by decreasing BWS, altering speed, increasing trunk control, decreasing manual assistance for limb control and increasing the stepping time on the treadmill per bout. All participants received the same number of sessions and spend approximately the same amount of time involved in training, although progression of training parameters was individualized. Immediately following step training on the treadmill, each participant engaged in 20 minutes of over ground training. Over ground training incorporated the use of assistive devices, but participants were otherwise bearing full weight on their lower extremities. A more detailed description of the training principles and parameters has been provided (Behrman and Harkema 2000; Behrman, Lawless-Dixon et al. 2005).

5.1.4 Proton Magnetic Resonance Imaging (^{1}H-MRI)

Proton magnetic resonance imaging (^{1}H-MRI) was used to determine a) maximum muscle CSA and b) T2 relaxation properties of the lower extremity muscles.

A 1.5 Tesla super conducting magnet scanner (Signa; General Electric)[2] was used to collect trans-axial images of the leg and thigh. The self-reported more involved leg of the SCI group and the right leg of control subjects was scanned. A standard (20cm long) lower extremity quadrature coil or body coil was employed for imaging. The extremity coil covered the length of the leg starting from above the lateral malleolus and extending to ~3 cm centimeters proximal to the superior patella. T1 weighted image of the leg with a pulse sequence of TR=300ms, TE= minimum, matrix = 128*128 and FOV = 16-30cm served as a coronal localizer for axial imaging (Figure 5-1). Trans-axial images for measurements of CSA and T2 relaxation properties were obtained using distinct NMR sequences.

5.1.4.1 Muscle cross sectional area: data collection and analysis

Spin echo or fat suppressed 3D SPGR imaging sequence was utilized with the following imaging parameters: acquisition matrix size, 256x256 to 256x192; field of view of 16cm to 32cm for the leg and 22cm to 40cm for the thigh; pulse repetition time, 51ms–300 ms; echo time, 10ms-27ms; slice thickness of 5-7mm; slice gap, 0-5mm.

The images were transferred to a silicon graphics UNIX workstation and fat-free maximal muscle CSA of lower extremity muscles was determined using a custom-designed interactive computer program as previously described (Elliott, Walter et al. 1997)**.** Briefly, multiple slices (8-20) of each muscle were outlined taking care to avoid fascia and blood vessels, manually thresholded to include pixels of similar signal intensity that represent muscle tissue and then segmented to determine the slice with maximum CSA. A unique feature of this software was its calculations for partial volume effects thereby yielding an accurate measure of muscle CSA. Maximum CSA of the quadriceps femoris (QF) and hamstring (HAMS) muscle groups in the

[2] GE Medical Systems global headquarters: Waukesha, Wisconsin

thigh, the soleus (SOL), medial gastrocnemius, lateral gastrocnemius (LG), tibialis anterior (TA) muscles in the lower leg and maximum CSA of the entire posterior compartment (PC) of the lower leg (that includes the tibialis posterior muscle) were calculated (Figure 5-2). In addition, maximum CSA of the ankle anti-gravity muscles [plantar flexors (PF)] was considered at the level of the lower leg where the SOL, MG and LG taken together resulted in the largest CSA.

5.1.4.2 T2 relaxation times: data collection and analysis

T2 weighted imaging using multiple slice spin echo sequence was performed with following imaging parameters TR = 2000ms; TE = 26, 52, 78 and 108ms; FOV = 16cm, slice thickness = 7mm, matrix of 256x128.

The T2 weighted images were transferred to a UNIX workstation and characteristic T2 relaxation time of the muscle and bone marrow were calculated using a custom designed software assuming a singe exponential decay with respect to the four imaging echo times. The T2 relaxation times were represented on resultant images called T_2 maps (Figure 5-3). Specific regions of interests (ROI) that avoid visible blood vessels in the SOL, MG, LG and TA muscles were identified in 8-10 T2 map slices and the average T2 of each muscle was subsequently calculated. T_2 times of bone marrow were measured as internal references to assess reproducibility of T2 values.

5.1.5 Proton Magnetic Resonance Spectroscopy (^{1}H-MRS)

Localized unsuppressed spectra were acquired from the soleus muscle to measure a) muscle lipid content b) T2 relaxation characteristics of muscle independent of fat.

For precise localization of volume of interest, a voxel (35mm thickness) was prescribed over the localized trans-axial image of the soleus muscle avoiding visible blood vessels and muscle fascia (Figure 5-4). In order to ascertain voxel position over the muscle without signal contamination from non-selected surrounding tissue, a phantom experiment was performed to

obtain an image of the prescribed voxel. Figure 5-5 shows a raw image of the voxel acquired at the 1.5T from a CuSO4 phantom (TR =2000ms, TE = 18, FOV =16cm). Note that the boundaries of the voxel are sharp and its dimensions the same as the prescribed voxel dimensions. An unsuppressed water stimulation acquisition mode spectroscopic sequence - STEAM (Bruhn, Frahm et al. 1991) sequence was used with four different echo times (13ms, 30ms, 60ms and 120ms), a TR of 6000ms, 4 scans, 512 points and 2500 Hz spectral width. In addition, a 32scan average was performed at the 120ms TE to estimate total lipid content and individual lipid components; intramyocellular (IMCL) and extramyocellular lipids (EMCL).

5.1.5.1 Muscle lipid: data analysis

A zero order phase correction was performed on the 32 scan raw spectrum and water and lipid spectral peaks were quantified using an Advanced Magnetic Resonance (AMARES) time-domain-fitting algorithm using jMRUI (Naressi, Couturier et al. 2001). Prior knowledge values were constructed from healthy controls for measuring water and lipid amplitudes. Whole lipid peak and water peak were identified at 1.5ppm and 4.7ppm (Figure 5-4). Once amplitudes from the water and whole lipid peak were calculated, the water peak was manually suppressed during data analysis. Lipid resonance peak was subsequently deconvoluted to estimate IMCL and EMCL at approximately 1.25ppm and 1.4ppm using the AMARES method (Figure 5-6). Thereafter, IMCL and EMCL lipid amplitudes were calculated from their spectral peaks and corrected for T_2 relaxation effects using T2 relaxation times of 85ms for IMCL and 75ms for EMCL. T2 relaxation times of IMCL and EMCL in the soleus muscle used in this study match with lipid T2 relaxation values reported in literature (Bruhn, Frahm et al. 1991; Szczepaniak, Babcock et al. 1999). In concurrence with other studies, the overall lipid, IMCL and EMCL content in our study were expressed as a ratio using the spectral water peak as an internal reference (Sinha, Dufour et al. 2002; White, Ferguson et al. 2006).

Uniqueness of this method to analyze fat was that it exclusively represents intramuscular fat. Studies in the past have quantified fat within muscle using imaging techniques. However, this method is plagued by the inclusion of inter-muscular fat (Elder, Apple et al. 2004) and requires a good amount of fat to adequately quantify it (Huang, Majumdar et al. 1994). Proton spectroscopy on the other hand, can yield a good lipid peak along with fat components from within the same muscles.

5.1.5.2 Muscle T2 relaxation times: data analysis

The T_2 relaxation time of soleus muscle independent of fat was calculated assuming a single exponential decay with respect to the four spectral echo times.

Mono-exponential decay versus multi-exponential decay curves: In this study, only four echo times were used to acquire both imaging and spectroscopy data, which consequently required assumption of a single exponential decay to calculate T2 relaxation times from each muscle. This might pose a potential limitation to data collection in our present study. Chances prevail that the single exponential decay curves might in fact be multi-exponential, with contributions from multiple T2 components. Various algorithms including the non-negative least squares (NNLS) are commonly used to decompose multiple component curves into its distinct components. However, for decomposition of the signal into its components using NNLS, several TEs are necessitated during data collection.

5.2 Animal Studies

5.2.1 Animals

Sixteen adult Sprague Dawley female rats (16 weeks of age, 228-260g; Charles River, NJ) either underwent a spinal cord contusion (n=8) or served as controls for wet lab procedures relevant to the study. Briefly, experimental rats were tested for outcome measures before injury and were followed up longitudinally for three weeks after injury. Animals were housed in a

temperature controlled room at 21 ^{0}C with a 12:12 hour light: dark cycle and were provided rodent chow and water ad libitum. All experimental procedures were performed in accordance with the U.S. Government Principle for the Utilization and Care of Vertebrate Animals by approval of the Institutional Animal Care & Use Committee at the University of Florida. At the end of the study, all animals were euthanized and hind limb muscles were extracted and processed for biochemical assays.

5.2.2 Gender Differences in Animal Models of SCI

Most studies pertaining to spinal cord injury research have used the female rat model of spinal injury. While studying both male and female models for muscle adaptations will assist in teasing out any gender differences in muscle adaptations, we have chosen to utilize female rats in the present work. One of the main reasons for our choice vests in remaining consistent with reports in literature and taking advantage of valid comparisons with published work by other investigators.

5.2.3 Spinal Cord Contusion Injury

Spinal cord contusion injury was produced using a NYU (New York University) impactor device. A 10g weight was dropped from a 2.5-cm height onto the T8 segment of the spinal cord exposed by laminectomy under sterile conditions. Animals received two doses of Ampicillin per day for 5 days, starting at the day of surgery. Procedures were performed under ketamine (100mg/kg)-xylazine (6.7mg/kg) anesthesia (Reier, Anderson et al. 1992; Thompson, Reier et al. 1992). Subcutaneous lactated Ringer's solution (5 ml) and antibiotic spray were administered after completion of the surgery. The animals were kept under vigilant postoperative care, including daily examination for signs of distress, weight loss, dehydration, and bladder dysfunction. Manual expression of bladders was performed 2-3 times daily, as required, and animals monitored for the possibility of urinary tract infection. Animals were housed

individually following surgery. At post-operative day 7, open field locomotion was assessed using the 21 Basso-Beattie-Bresnahan (BBB) locomotor scale (Basso, Beattie et al. 1995) and animals that did not fall within a preset range (0-7) were excluded from the study. After baseline measurements before injury, rats underwent once weekly MRS measurements for three weeks starting at week one after injury.

5.2.4 Experimental Electrical Stimulation Protocol

An electrical muscle stimulation protocol was adopted to determine the mitochondrial oxidative capacity of the rat hind limb muscle *in-vivo*. Animals were anesthesized using gaseous isoflurane in oxygen (3% box induction), and maintained at 0.5%-2.5% during the MR procedures. After shaving the limb and cleaning it with alcohol, an oval surface coil tuned to ^{31}P (190.5 MHz) was placed over the belly of the gastrocnemius muscles. A ^{1}H surface coil was placed underneath the hind limb to perform shimming. Two needle electrodes were placed subcutaneously – one over the region of the third lumbar vertebrae and the other land marked over the greater trochanter - to stimulate the hind limb plantarflexor muscles via stimulation of the sciatic nerve (Figure 5-9). Electrical stimulation was carried out for four to six minutes to deplete PCr by 30 to 40%. A Grass Stimulator (Quincy MA) with a Grass Model SIU8T stimulation isolation unit (Grass Instruments, West Warwick, RI) was used to deliver a monophasic, rectangular pulse with a 1ms pulse duration, 1Hz frequency and 10V. Following the electrical stimulation, the muscle was allowed to recover for twenty minutes. Conceptually, electrical stimulation depletes phosphocreatine (PCr) from the contracting muscle and the rate of PCr recovery is measured to determine skeletal muscle oxidative capacity. During the entire duration of stimulation and recovery, spectra were collected. No attempts were made to synchronize the radiofrequency pulse with the muscle stimulation. The FIDs were multiplied by

an exponential corresponding to a 25Hz line broadening. Vital signs of the animal were monitored throughout the experimental procedure.

5.2.5 Phosphorus Magnetic Resonance Spectroscopy (^{31}P-MRS): Data Collection

All data was collected using a high magnetic field strength Bruker 11Tesla/470 MHz spectrometer. A 1.5 x 1.7 cm oval surface coil tuned to ^{31}P (190.5 MHz) was placed over the belly of the gastrocnemius muscle. A 3-cm standard ^{1}H surface coil was placed underneath the hind limb to perform shimming and the animal's hind limb was extended such that the calf muscles were centered over the surface coil. For the baseline ^{31}P MRS spectra data, spectra were acquired with a 50 μs square pulse, a TR of 2s, spectral width of 10,000 Hz, 150 averages and 8000 complex data points. For the ^{31}P kinetic data, spectra were averaged into 20s bins and acquired at rest (5 min), electrical stimulation (4-6 min), and recovery (20 min); thus amounting to a total of 93 fids. The partially relaxed spectra were then calibrated by comparison with fully relaxed spectra (acquired at TR of 15seconds) to determine correction factors (CF).

5.2.5.1 ^{31}P-MRS spectral analysis at rest

Resting spectra yields measures of basal $P_{i\ and}$ PCr concentration from the resting gastrocnemius rat hind limb muscles. The spectra were manually phased, and the areas of the β ATP, P_i, and PCr peaks determined by area integration. The enzymatically determined ATP concentration in frozen muscle tissue was equated with the integral of the β-ATP. Equating tissue measurements of ATP under the β-ATP peak is established as valid for skeletal muscle (Hitchins, Cieslar et al. 2001). Pi and PCr concentrations were determined by using β-ATP as an internal standard and after accounting for correction factors (CF) as follows:

$$[\text{Metabolite}]\ (\mu\text{mol/g wet wt}) = CF_{\text{Metabolite}} \times \frac{\text{Integral}_{\text{Metabolite}}}{\text{Integral}_{\beta\text{-ATP}}} \times [\text{ATP}]\ (\mu\text{mol/g wet wt}) \quad (5\text{-}1)$$

ATP concentration in gastrocnemius muscle obtained by ATP assay was 4.6 ± 0.3 μmol/g wet wt and is similar to ATP concentrations in rat gastrocnemius muscle reported in literature (Authier, Albrand et al. 1987; Hitchins, Cieslar et al. 2001; Gigli and Bussmann 2002). Similarly, our correction factors for Pi and PCr were 1.69 and 1.40 respectively and match well with published values from rat gastrocnemius muscle (Mizobata, Prechek et al. 1995). Intracellular pH were calculated from the chemical shift of the P_i peak relative to PCr using the equation, $pH = 6.75 + \log [(\delta - 3.27) / (5.69 - \delta)]$, where δ is the chemical shift of the P_i peak in ppm. The cystolic phosphorylation potential was calculated in reciprocal form as [Pi][ADP]/[ATP] since the phosphorylation potential itself is not normally distributed. Free cystolic ADP was calculated from the creatine kinase equilibrium reaction as previously described (Mizobata, Prechek et al. 1995; Thompson, Kemp et al. 1995; Pathare, Vandenborne et al. 2007):

$$[\text{ADP}] = \{[\text{free creatine}][\text{ATP}]\}/\{[\text{PCr}][\text{H+}][K_{eq}] \quad (5\text{-}2)$$

where the free creatine was quantified by subtracting the PCr content obtained by ^{31}P-MRS from the total creatine content (42.2mM) determined biochemically. Intracellular Mg concentration and equilibrium constant (K_{eq}) of the creatine kinase reaction were assumed as 1mM and 1.66×10^9 respectively for mammalian skeletal muscle (Veech, Lawson et al. 1979).

5.2.5.2 ^{31}P-MRS spectral analysis of electrical stimulation protocol

The electrical stimulation protocol data yields kinetic measurements of PCr. Dynamic changes in PCr levels were measured using complex principal component analysis (Elliott,

Walter et al. 1999). Recovery data were fitted to a single exponential curve, and the pseudo first-order rate constant for PCr recovery (k_{PCr}) was determined (Meyer 1988). The maximal rate of PCr resynthesis, a measure of mitochondrial oxidative capacity ($V_{max\text{-}lin}$) was calculated based on k_{PCr} and PCr rest ($V_{max\text{-}lin} = k_{PCr} . [PCr]_{rest}$) (Walter, Vandenborne et al. 1997). Initial rates of PCr recovery (V_{meas} mM/min, a direct measure of mitochondrial ATP synthesis) was determined from the first three to four data points in recovery; depending upon the best linear curve fit. The initial rate of PCr resynthesis was also extrapolated from the product of k_{PCr} and amount of PCr depletion (ΔPCr). Thus, V_{ex} (mM/min) = k_{PCr}. ΔPCr (Walter, Vandenborne et al. 1997).

Rates of PCr depletion at onset of stimulation (V_{dep} mM/min, a measure of ATP demand) were determined from the first three to nine data points (first through three minutes) of PCr declines during stimulation.

The maximum oxidative ATP synthesis rate (Q_{max}), which is a function of intrinsic mitochondrial content and enzyme activity, oxygen and substrate supply to the mitochondrion, and cytosolic redox state (Kemp, Sanderson et al. 1996), was calculated from the known hyperbolic relationship between PCr resynthesis and cytosolic free [ADP] and from PCr resynthesis and [ADP][Pi]/[ATP].

$Q_{max\text{-}ADP}$ = Vmeas (1 + Km/[ADP]) mM/min, where Km is the Michaelis constant and is assumed as 50 μM for rat leg muscle (Thompson, Kemp et al. 1995). (5-3)

$Q_{max\text{-}[ADP][Pi]/[ATP]}$ = Vmeas (1 + Km/[ADP][Pi]/[ATP]) mM/min, where Km is assumed as 0.11 mM. (5-4)

5.2.6 Biochemical Assays

Animals were sacrificed at the end of the experiments and gastrocnemius muscle excised and snap frozen at -80^{0}C for subsequent biochemical quantification.

5.2.6.1 ATP measurements

ATP was measured as previously described (Hitchins, Cieslar et al. 2001; Gigli and Bussmann 2002; Pathare, Vandenborne et al. 2007). Frozen gastrocnemius muscle was ground to fine powder using a mortar and pestle under dry-ice. 100mg of the tissue was homogenized for 30seconds with a Mini-bead-beater in a plastic Eppendorf tube containing beads and ice-cold 0.9% perchloric acid (5v/w). The sample was then centrifuged at 9000 g for 15 minutes at 4^0C (~4000rpm). The supernatant was extracted and added to 4M KOH (1.125v/w) and centrifuged again for 5 minutes at 4^0C and 9000 g. The supernatant was frozen at -80^0C and processed for ATP measurements with an ATP assay kit (Sigma) using a luminometer (Biotek Instruments, CA).

5.2.6.2 Total creatine measurements

Total muscle creatine content was determined using the diacetyl/α-napthtol assay ((De Saedeleer and Marechal 1984; Vandenberghe, Gillis et al. 1996; Tarnopolsky and Parise 1999). Approximately 10-15 mg (average 11.55 ± 2.25 mg) of muscle tissue was cut and placed in a microfuge tube, and then placed in a vacuum centrifuge (Savant ISS110 SpeedVac^{TM} Concentrator, Thermo Scientific, Milford, MA) to be spun for 18-24 hours. After sufficient muscle drying, the samples were then placed in an ultra-low freezer at -80°C. Dried muscle was powdered by grinding on a porcelain plate with a pestle. Connective tissue was removed and discarded, whereas powdered muscle was placed into pre-weighed microfuge tubes and dry weight determined. Powdered muscle (average 2.27 ± 0.44 mg) was extracted in a 0.5 M perchloric acid/1 mM EDTA solution at a relative ratio of 800 µl per 10 mg powdered muscle on ice for 15-minutes, while periodically vortexing. Samples were then spun at 15,000 rpm at 4˚C for 5-minutes. The supernatant was transferred into a microfuge tube and neutralized with 2.1 M $KHCO_3$ / 0.3 M MOPS solution at a ratio of 1:5 and then centrifuged again at 15,000 rpm for 5-

minutes. The resulting supernatant was then stored at -80˚C for future use. In order to determine muscle total creatine concentration, 40 µl of the supernatant from the above reaction was combined with 140 µl ddH_2O and 20 µl 0.4 N HCl and heated at 65˚C for 10-minutes to hydrolyze phosphate groups. The solution was then neutralized with 40 µl of 2.0 N NaOH and analyzed as described above.

TCr and ATP levels were determined in µmol (g wet weight)$^{-1}$ and mmol (l tissue)$^{-1}$, assuming a muscle density of 1.06 gml^{-1}. These were converted to mmol l^{-1} intracellular water assuming a cellular water fraction of 0.83 in the rat gastrocnemius muscle (Veech, Lawson et al. 1979; Cieslar, Huang et al. 1998).

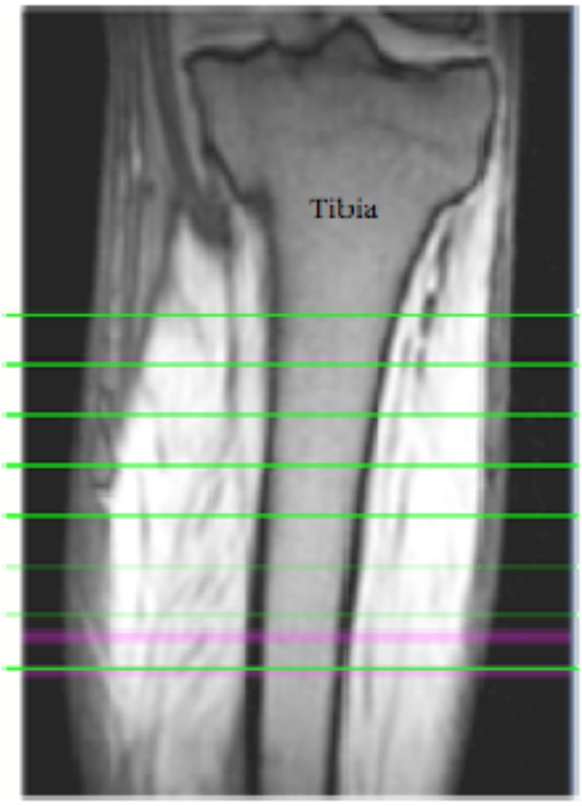

Figure 5-1. Representative ^{1}H-MRI coronal image of the calf muscles from a healthy control at 1.5T. Trans-axial images were obtained along the length of the coronal image (represented by horizontal green lines)

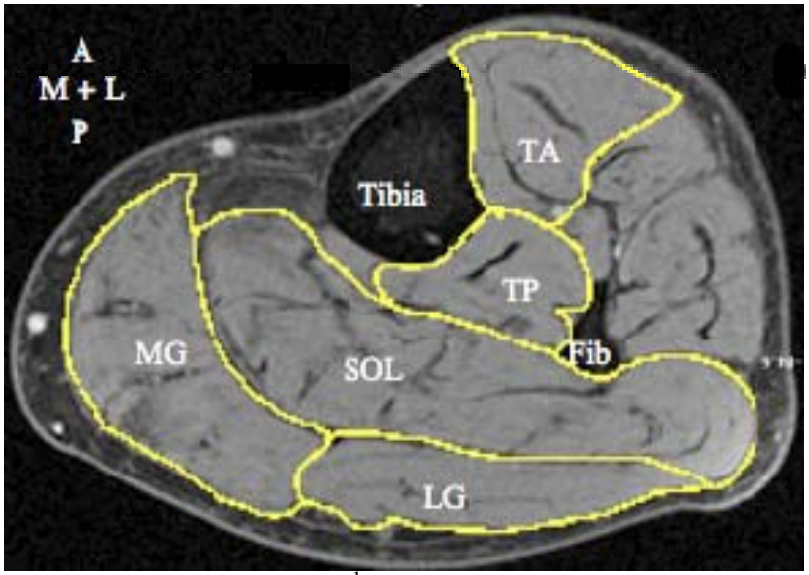

Figure 5-2. Representative ^{1}H-MRI trans-axial image of the calf muscles from a healthy control at 1.5T. A 3D fast gradient echo imaging sequence was used in a 1.5Tesla magnet. (A= anterior; P=posterior; M= medial; L=lateral)

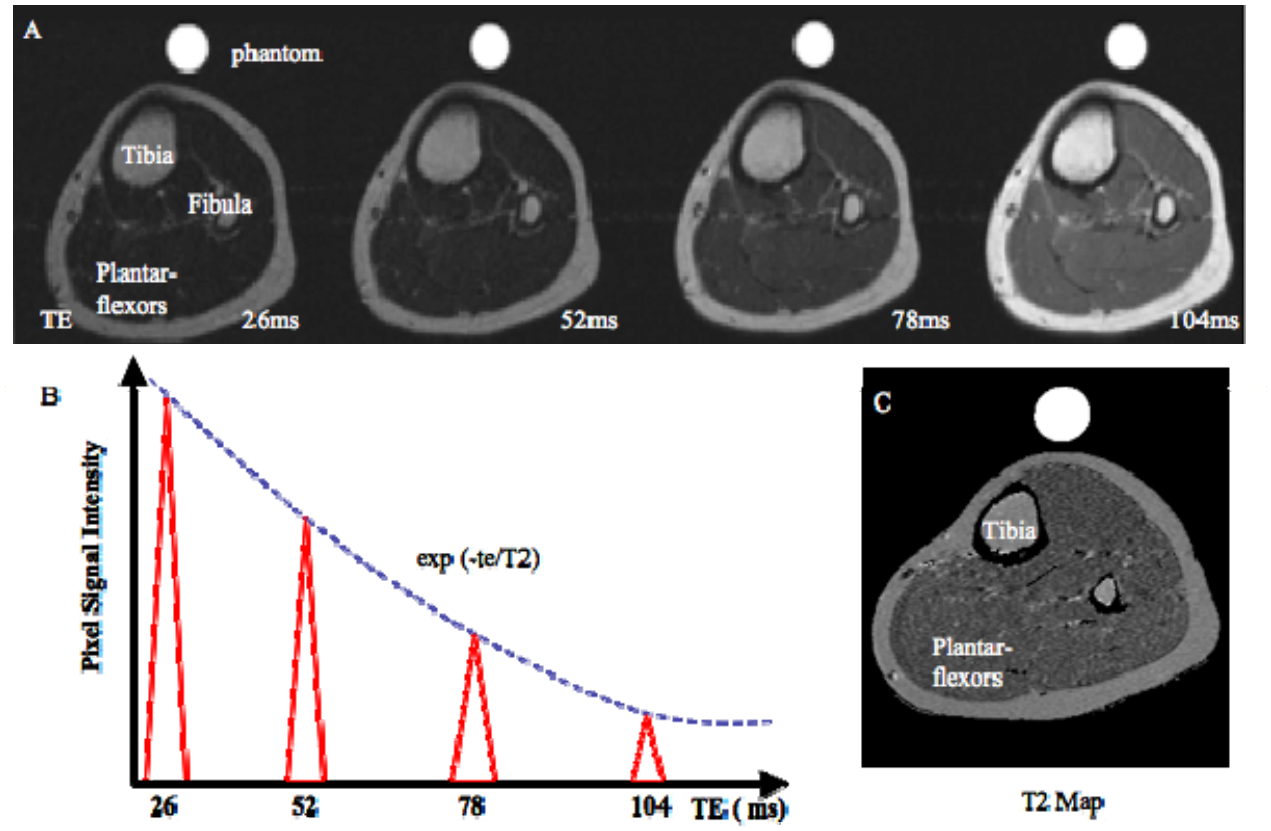

Figure 5-3. A) Representative ^{1}H-MRI T2 weighed images of the calf muscles obtained at four different echo times. B) Individual pixel signal intensities were fitted to an exponentially decaying curve along four different echo times (TE) C) Representative T2 map of the T2 weighted images acquired in A.

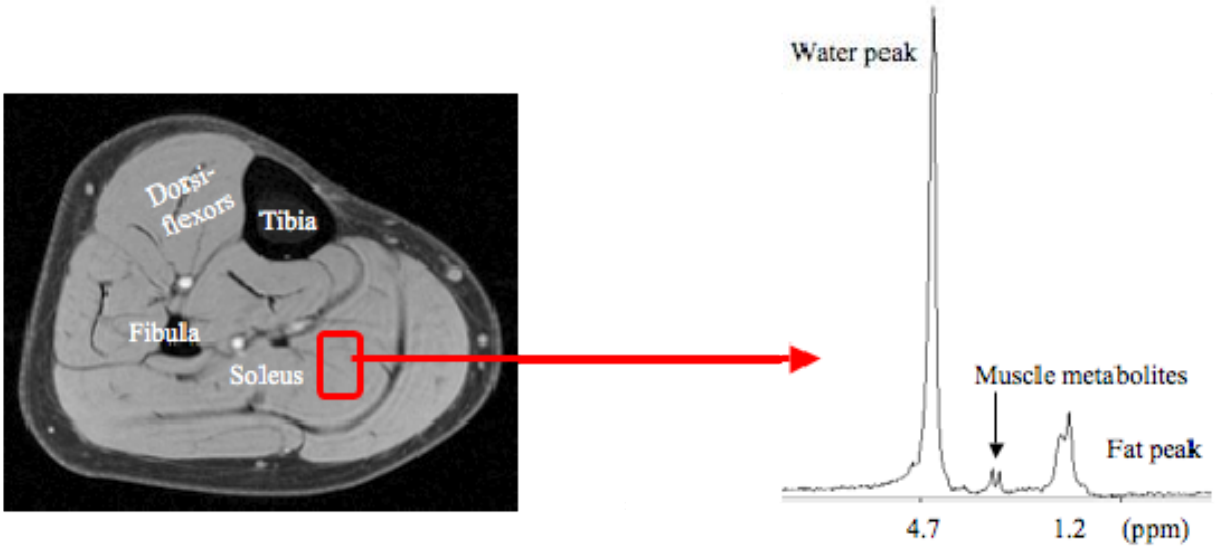

Figure 5-4. Representative trans-axial image presents a voxel prescribed over the soleus muscle and a resultant ^{1}H-MR spectrum with water and fat peaks.

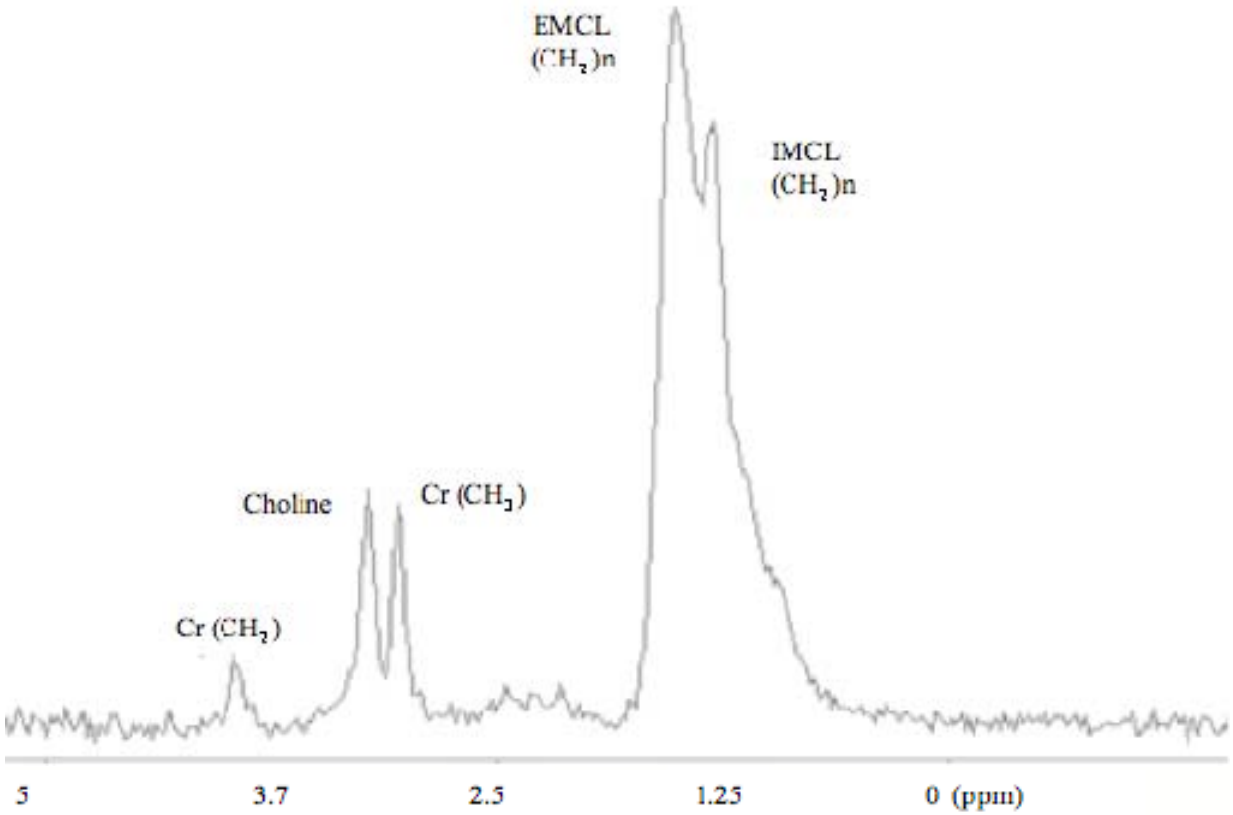

Figure 5-5. Decomposition of a lipid peak obtained from a healthy soleus muscle into its intramyocellular and extramyocellular components using jMRUI.

Figure 5-9. Experimental-setup for electrical stimulation protocol during ^{31}P-MRS data acquisition.

CHAPTER 6
EXPERIMENT ONE - LOWER EXTREMITY MUSCLE CROSS-SECTIONAL AREA AFTER INCOMPLETE SPINAL CORD INJURY

6.1 Summary

The purpose of this study was to: a) To quantify skeletal muscle size in lower extremity muscles in persons after incomplete-SCI, b) to assess differences in muscle size between involved lower limbs, c) to determine the impact of ambulatory status (using wheelchair for community mobility versus not using a wheelchair for community mobility) on muscle size after incomplete-SCI, d) to determine if differential atrophy occurs among individual muscles after incomplete-SCI. Seventeen persons with incomplete-SCI and 17 age, gender, weight and height matched non-injured controls participated from the university research setting. Maximum cross sectional area (CSA) of individual lower extremity muscles [soleus, medial gastrocnemius, lateral gastrocnemius, tibialis anterior, quadriceps femoris and hamstrings] was assessed by Magnetic Resonance Imaging. Overall, subjects with incomplete-SCI demonstrated significantly smaller (24% - 31%) average muscle CSA in affected lower extremity muscles as compared to control subjects ($P<0.05$). Mean differences were highest in the thigh muscles (~31%) compared to the lower leg muscles (~25%). No differences were noted between the self-reported more- and less-involved limbs within the incomplete-SCI group. Dichotomizing the incomplete-SCI group revealed significantly lower muscle CSA values in both the wheelchair [range = 21% - 39%] and non-wheelchair groups [range = 24% - 38%]. In addition, the wheelchair group exhibited significantly greater plantar flexor muscle atrophy compared to the dorsi-flexors, with maximum atrophy in the medial gastrocnemius muscle (39%). Our results suggest marked and differential atrophic response of the affected lower extremity muscles that is seemingly impacted by ambulatory status in persons with incomplete-SCI.

6.2 Introduction

An emerging trend in the care and treatment of persons after spinal cord injury (SCI) is the increased proportion of persons diagnosed with incomplete injuries. Persons with incomplete-SCI exhibit variable paralysis and paresis of affected muscles, typically resulting in impaired motor performance and varying degrees of functional limitations (Subbarao 1991; Tang, Tuel et al. 1994; Burns, Golding et al. 1997). Interestingly, although incomplete-SCI constitutes ~51% of all new spinal injuries, the majority of human and animal research related to physiological and morphological adaptations following SCI has focused on subjects with complete injuries. As such, a large body of literature exists that describes skeletal muscle adaptations after this type of injury (Baldi, Jackson et al. 1998; Hopman, Dueck et al. 1998) with few data describing adaptations within affected skeletal muscle after incomplete injuries. Similar to persons with complete-SCI, persons with incomplete-SCI exhibit a variety of clinically relevant motor and functional deficits, including local muscle fatigue, weakness of affected muscles (Sloan, Bremner et al. 1994; Johnston, Finson et al. 2003) and diminished capacity to ambulate(Waters, Adkins et al. 1994; Ulkar, Yavuzer et al. 2003). We recently demonstrated that after chronic upper motor lesions and incomplete-SCI, both knee extensor and plantar flexor skeletal muscles generate ~70% less peak torque (Jayaraman, Gregory et al. 2006). Other studies have shown a significant reduction in ambulatory capacity, with a reduced gait speed, step frequency and stride length. Despite such obvious motor dysfunction, no studies have documented the extent of muscle atrophy in paralyzed skeletal muscle following incomplete-SCI in humans. Given that muscle atrophy relates strongly to compromised muscle strength (Berg, Dudley et al. 1991; Ploutz-Snyder, Tesch et al. 1995; Vandenborne, Elliott et al. 1998; Stevens, Walter et al. 2004) as well as locomotor ability,(Visser, Kritchevsky et al. 2002; Visser, Goodpaster et al. 2005) an

in-depth understanding of the extent of impairment in this population would be valuable to the field of rehabilitation research.

Persons with incomplete-SCI constitute an extremely heterogeneous group. For example, people after this type of injury exhibit a continuum of ambulatory abilities ranging from being completely wheelchair dependent to nearly normal walking without the use of assistive devices. Consequently, the mechanical loading and activation of the affected lower extremity muscles is extremely variable (Melis, Torres-Moreno et al. 1999). Given that forced inactivity of lower extremity muscles (i.e. immobilization, limb suspension) results in differential skeletal muscle atrophy (Adams, Hather et al. 1994; Adams 2002; Alkner and Tesch 2004) one might expect variable patterns of muscle adaptations in persons after incomplete-SCI. Accordingly, we sought to examine the morphological characteristics of lower extremity skeletal muscles in persons with incomplete-SCI. Specifically, the purpose of our study was four-fold: 1) to compare lower extremity muscle maximum cross sectional area (CSA) in persons with incomplete-SCI to a group of age, gender, height and weight matched controls 2) to make comparisons of maximum muscle CSA between the self-reported more and less involved limbs within a group of persons with incomplete-SCI 3) to evaluate whether ambulatory status (i.e. using wheelchair for community mobility versus not using a wheelchair for community mobility) influences lower extremity skeletal muscle CSA after incomplete SCI 4) to compare the magnitude of atrophic response a) between the flexor and extensor muscles about the knee and ankle, b) between proximal and distal anti-gravity extensor muscles, and c) among individual ankle plantar flexor muscles.

6.3 Methods

We performed a case-control study in which the lower extremity maximum muscle CSA of persons with incomplete-SCI was compared with maximum muscle CSA of age, gender, weight and height matched non-injured controls. To address the impact of ambulatory status on skeletal muscle size, we dichotomized our incomplete-SCI subjects into those who did not have upright mobility in the community, but demonstrated ambulatory ability through use of a wheelchair (W/C group) and those who did not use a wheelchair for community mobility (non-W/C group).

6.3.1 Subjects

Persons with incomplete-SCI: A convenient sample of seventeen persons (2 women, 15 men; 13±9 months post-injury) with incomplete-SCI (39±15 yr, 76±13 kg, 178±10 cm) volunteered to participate in the study. Of these, 10 subjects were studied at the University of Florida, Gainesville, Florida; and 7 subjects were studied at the University of Georgia, Athens, Georgia. All participants 1) had a diagnosis of traumatic SCI at cervical or thoracic levels (C4-T12) resulting in upper motor neuron lesions in the lower extremity, 2) had a history of SCI as defined by the American Spinal Injury Association (ASIA) Impairment Scale categories C or D, and 3) had a medically stable condition at the time of testing. Seven of the persons with incomplete-SCI used a wheelchair, two subjects used forearm crutches, and six used a single-point cane for community ambulation (Table 6-1).

Controls: Seventeen persons (2 women, 15 men; 39 ± 12 yr, 78 ± 12 kg, 178±8 cm) volunteered to serve as control subjects. These subjects were matched to incomplete-SCI subjects on the basis of age, gender, and height and body mass. Though large demographic variability existed among subjects in both the incomplete-SCI and control groups, each control person was closely matched (age ± 7 yr, height ± 10 cm, and body mass ± 8 kg) to the corresponding person with incomplete-SCI. Control subjects were recreationally active, but not

engaged in any rigorous exercise program, and were recruited from the Gainesville, FL community.

6.3.2 Maximum Muscle Cross-sectional Area

Proton magnetic resonance imaging (MRI) was used to determine maximum muscle CSA of the lower extremity. MRI for all subjects was performed specifically for the study. Details of the MRI procedures are described in Chapter 5 (section 5.1.4.1). Figure 6-1 illustrates representative trans-axial proton magnetic resonance images of patient and control subjects obtained at 1.5Tesla magnetic field.

6.3.3 Data Analysis

Independent samples t-tests were employed to determine if differences existed in the demographic characteristics (age, height and body weight) and to compare the maximum muscle CSA of the muscles of interest in the pooled incomplete-SCI (n=17) and control groups (n=17). For skewed data, distribution-free Mann Whitney tests were used to compare muscle CSA between controls and persons with incomplete-SCI. The self reported more- and less-involved limbs of persons with incomplete-SCI (n=7) were compared using a two-related sample Wilcoxon test. To determine the impact of ambulatory status on maximum muscle CSA we compared the mean CSA of the above-mentioned muscles in the W/C (n=7) and non-W/C groups (n=10) with their corresponding matched controls using Mann Whitney test. In all analyses involving comparisons for incomplete-SCI with controls, a unidirectional hypothesis (incomplete-SCI CSA < control CSA) was tested. Further, relative differences between the extensor and flexor muscles about the knee and ankle were determined by intra-compartment (PF:TA, QF:HAMS) ratios. In addition, relative differences between proximal and distal anti-gravity muscles were compared using inter-compartment (QF:PF) ratios. Lastly, differential atrophy among the specific plantar flexor muscles was determined by normalizing each

individual plantar flexor muscle to the maximum total posterior compartment (PC) muscle CSA (SOL:PC, MG:PC, LG:PC). The analyses for relative atrophy between muscles were done both on the pooled incomplete-SCI groups versus controls and the wheelchair versus non-wheelchair groups. After obtaining the proportions, a proportionality test was run (Ho: $\prod 1 = \prod 2$) (Alan Agresti 1997) to determine if statistical differences existed between the groups. SPSS for Windows (Version 11.0.1)[3] was utilized for all statistical analyses. Alpha level was set at 0.05 and Dunn-Bonferroni corrections for multiple comparisons were made where appropriate). Percent differences in maximum muscle CSAs between groups were calculated by taking the average of the individual percent differences between persons with incomplete-SCI and their corresponding control subjects.

6.4 Results

Demographic data: No differences existed between the incomplete-SCI and control group with respect to age, height or weight ($p \geq 0.562$).

Maximum muscle CSA after incomplete-SCI: Lower extremity muscle size in the pooled incomplete-SCI subjects was significantly smaller than the control group for all of the tested muscles ($p \leq 0.004$). Mean differences in muscle CSA ranged from 24% (TA) to 31% (QF) in the incomplete-SCI group relative to controls. Muscle specific CSA data are presented in Figures 6-2A and 6-2B.

Bilateral differences in maximum muscle CSA after incomplete-SCI: No significant differences in maximum muscle CSA were found between the self-reported more-involved versus the less-involved limbs within the incomplete-SCI subjects for any of the muscles tested ($p \geq 0.473$).

[3] SPSS Inc, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606

Impact of ambulatory status on maximum muscle CSA after incomplete-SCI*:* Our results showed that persons with incomplete-SCI in both the W/C and non-W/C group showed a lower skeletal muscle size as compared to controls. As shown in Table 2, subjects in the W/C group had significantly smaller muscle CSA values for all of the anti-gravity muscles (i.e. SOL, MG, LG, QF) relative to their corresponding control group ($p \leq 0.048$). Mean differences in lower extremity muscle CSA in the W/C group ranged from 21% (QF, TA) to 39%. Interestingly, neither TA ($p=0.064$) nor the HAMS ($p=0.120$) were significantly different in the W/C group when compared to matched controls. The non-W/C i-SCI group also showed significant differences in muscle CSA values relative to control subjects. With the exception of the MG, all of their lower extremity muscle CSA values were significantly smaller than those measured in the control group ($p \leq 0.021$). As shown in Table 2, mean differences in muscle CSA in the non-W/C group ranged between 24% (SOL) and 38% (QF) relative to the control group.

Differential atrophy after incomplete-SCI: Comparisons in the magnitude of atrophy across affected lower extremity muscles revealed no differential atrophy between extensor and flexor muscle groups about the ankle (PF:TA, $p = 0.433$) or knee (QF:HAMS, $p = 0.769$) in the pooled incomplete-SCI group and control groups (Table 3). However, as shown in Figure 6-3, similar comparisons suggest greater relative atrophy of the anti-gravity muscles in the leg in the W/C group compared to the non-W/C group (PF: TA, $p = 0.043$). When examined separately, the proportion of the overall plantar flexor CSA occupied by SOL or the LG was not different between the pooled incomplete-SCI and control groups (Table 3). However, the proportion of overall CSA occupied by the MG was significantly smaller in the W/C group relative to the non-W/C group (MG:PC, $p = 0.002$) (Figure 6-4). Lastly, the relative ratios between proximal and distal anti-gravity muscle CSA (QF:PF) were similar for all comparisons made ($p \geq 0.273$).

6.5 Discussion

The findings of our study indicate marked atrophy of lower extremity muscles following incomplete-SCI. Overall, subjects with incomplete-SCI demonstrate a 24% - 31% smaller average muscle CSA in affected lower extremity muscles as compared to control subjects. Mean differences were highest in the thigh muscles (~31%) compared to the leg muscles (~25%), with no differences noted between the self-reported more- and less-involved limbs within the incomplete-SCI group. Dichotomizing the incomplete-SCI group into a W/C and non-W/C users revealed that both the W/C and non-W/C groups had a significantly lower muscle CSA than their respective control groups. In addition, the W/C group exhibited greater atrophy in their ankle plantar flexor muscles compared to the dorsi-flexors, suggesting a preferential atrophic response in the anti-gravity muscles.

The magnitude of atrophy in our subjects after incomplete-SCI is much less than that reported in persons following complete-SCI. An overall 46% decline in average CSA of the lower extremity muscles in persons after complete-SCI has been reported 24 weeks after injury; with decreases in the SOL (68%), gastrocnemius (54%), TA (20%), QF (42%) and HAMS (44%) muscles reported relative to controls (Castro, Apple et al. 1999). These values (except for the TA muscle) are approximately twice that seen in the present study. One of the main reasons is likely related to the partial sparing of voluntary motor control following motor incomplete-SCI. Skeletal muscle atrophy following SCI is a result of primary injury to motor neurons in the spinal cord and concurrent inactivation of affected skeletal muscle along with subsequent changes in muscle length and mechanical loading conditions (Gordon and Mao 1994). Fractional presence of neural inputs to the affected muscle allows for variable activation of lower extremity musculature after incomplete-SCI. In fact, research subjects in our study were typically able to load their lower extremities during transfers and/or ambulate with crutches or a cane.

An interesting finding in the present study was the significant difference in lower extremity muscle CSA in both the W/C and the non-W/C incomplete-SCI groups. When compared to the control group, the W/C group showed atrophy in all the anti-gravity muscles. The non-W/C subjects demonstrated a similar pattern, with the exception that no differences were seen in the MG. One likely explanation for the atrophy seen in both the incomplete-SCI groups is that persons who use assistive aids (cane, crutches, etc) for ambulation transfer much of the weight bearing demands of daily activities to their upper extremities (Lee and McMahon 2002; Mulroy, Farrokhi et al. 2004). Consequently, weight bearing through the affected lower extremities is reduced. This reduced use and activation of the affected lower extremity muscles triggers a cycle of added muscle atrophy and further dependence on assistive devices. In fact, in a recent study, Clark et al. demonstrated reductions in leg muscular activity after use of a crutch or walker for ambulation in able-bodied subjects (Clark, Manini et al. 2004). The authors found that the knee extensor (vastus lateralis) and ankle plantar flexor muscles (soleus) incurred maximum decreases in muscle activity thereby suggesting a predisposition of the anti-gravity muscles to dysfunction following unloading. This finding is consistent with both animal and human models of unweighting (Thomason and Booth 1990; Adams, Caiozzo et al. 2003).

Unlike anti-gravity muscles, the proximal and distal flexors in the W/C and non-W/C groups showed varied findings. The ankle dorsi-flexor muscles showed significant differences in CSA in the non-W/C (~26%), but not in the W/C group (~21%). This finding seems counter intuitive given the perceived differences in the pattern of muscle activation between W/C and non-W/C users. However, it has been reported that anti-gravity extensor muscles contribute much more than the flexors toward an efficient gait (Saunders JBD 1953; Sutherland, Cooper et al. 1980; Basmajian JV 1988). Therefore, the discrepancy noted in the ankle and knee flexor

muscle response between the two groups is probably not related to activation patterns during gait. Interestingly, unlike the W/C group (1/7), a greater proportion of the subjects in the non-W/C group (5/10) were fitted with ankle-foot orthosis for daily use (Table 6-1). This characteristic may serve to explain the greater magnitude of atrophic response in the non-W/C group given the limited dorsi-flexion ROM allowed with the orthosis. However, the use of an orthosis did not necessarily correspond with lower extremity motor scores (LEMS, Table 6-1) in our incomplete-SCI groups. As such, we cannot fully explain the dorsi-flexor atrophic response in the non-W/C group.

When the pooled subject data were examined, the proportion of individual plantar flexor muscles relative to the compartments they occupy was consistent between the incomplete-SCI and control groups. This finding suggests that all the muscles studied underwent similar relative amounts of atrophy, independent of mobility status. However, when the W/C and non-W/C groups were examined individually, the MG muscle relative to the posterior compartment in the W/C group showed a differential atrophy compared to the other groups. Similarly, though no differences existed between the PF to TA ratios in the pooled incomplete-SCI and control groups, significantly lower PF to TA ratios are noted in the W/C group versus the non-W/C group suggesting more overall atrophy of the plantar flexors in the W/C group (~32% versus 21% in the W/C versus non-W/C, respectively). The most intuitive explanation for this finding would be the greater relative loading imposed on this muscle group during an upright (non-W/C) versus a seated posture (W/C). Previous studies have documented that the ankle plantar flexors are critical during locomotion and generate a majority of the propulsive forces necessary for efficient walking (Sutherland, Cooper et al. 1980; Basmajian JV 1988). Since walking is comparatively more compromised in persons who use a wheelchair versus a cane or crutch for

ambulation, mechanical loading via weight bearing on the paralyzed muscles is less. In addition to differential loading, a prolonged flexed position of the knee during use of a wheelchair might shorten the gastrocnemius muscle at the knee joint resulting in greater plantar flexor muscle atrophy relative to the non-W/C group.

In contrast to differences in leg muscle atrophy, differences in the thigh muscle CSA in the W/C and the non-W/C group were not significant. The QF to HAMS ratio was similar in both the i-SCI groups, implying relatively similar atrophy of the thigh musculature independent of ambulatory status. Lastly, the degree of atrophy between the anti-gravity proximal and distal extensor muscles (QF:PF) was also similar. Collectively, our results suggest that although using a cane or crutch for ambulation might attenuate the atrophic response of the ankle plantar flexor muscles (primarily the MG), yet marked adaptations are seen throughout the entire lower extremity after incomplete-SCI.

The atrophic response in persons after incomplete-SCI as described in this study most likely has dramatic functional implications. Previous studies have shown that decreases in muscle CSA are strongly related to impaired muscle strength (Ploutz-Snyder, Tesch et al. 1995; Vandenborne, Elliott et al. 1998; Stevens, Walter et al. 2004). In addition, muscle strength after incomplete-SCI plays an important role in functional walking performance (Kim, Eng et al. 2004). Also, gains in skeletal muscle size have been associated with improvements in motor function. For example, in a study assessing motor and sensory recovery following incomplete-SCI, hypertrophy of the partially innervated skeletal muscles was suggested as one of the factors that could account for the motor recovery following rehabilitation (Waters, Adkins et al. 1994). As such, if specific deficits associated with incomplete-SCI can be discerned, efficient strategies can be designed for functional rehabilitation.

Lastly, there are some shortcomings in our study. Subjects with i-SCI were, on an average, 13 months post injury (range, 5-37 months). Motor recovery following ISCI is a continuous process that reaches a plateau around one year post-injury, with a significantly slower rate of recovery in the second half year interval following injury (Waters, Adkins et al. 1994). As a result, subjects with i-SCI in this study were at slightly different stages of motor recovery and not necessarily in a steady condition (Waters, Adkins et al. 1994). Furthermore, our subjects underwent routine rehabilitation treatment prior to participation in the study. Therefore, it is possible that the true values of actual atrophy might have been underestimated. In addition, we cannot definitely confirm the subjects' pre-injury CSA values to be similar to the control group. However, our control subjects were matched to gender, age height and weight and have comparable CSA values to controls in other human studies (Castro, Apple et al. 1999). Despite these limitations, this study provides unique findings regarding the impact of ambulatory status on muscle CSA in individuals with incomplete-SCI.

In conclusion, this study demonstrates that incomplete-SCI is associated with significant muscle atrophy in the affected lower extremity that is uniform between limbs and somewhat influenced by mobility status. Interestingly, the majority of therapeutic approaches for improving locomotor performance in subjects with incomplete-SCI are compensatory rather than physiologically based. As such, persons after incomplete-SCI are often left with significant motor deficits despite long-term therapeutic intervention. With the increasing prevalence of persons living with incomplete-SCI, there is an urgent need to develop appropriate therapeutic techniques with the goal of maximizing motor recovery thereby reducing disability. When developing therapeutic interventions to enhance functional recovery after SCI, an understanding of the underlying physiology of muscular responses that occur after this type of injury may

promote different intervention strategies that rely less heavily on compensatory rehabilitation. This study will provide a foundation from which the relationship between muscle size and function in this population can be further explored. Future research efforts can be directed towards understanding relationships between physiological deficits in skeletal muscle following SCI and parameters of functional ability like walking balance, speed and muscle strength.

Table 6-1. Characteristics of subjects after incomplete-SCI.

	Months after injury	Level of injury	ASIA classification	Mobility status	Orthosis	LEMS
S1	5	C5	D	W/C	NA	35
S2	10	C5	D	Forearm crutch	AFO	40
S3	13	C5	D	Single point cane	AFO	43
S4	15	C6	C	W/C	NA	35
S5	16	C5	C	W/C	Bil-AFO	33
S6	20	T1	D	Single point cane	NA	44
S7	37	T1	D	Bilateral forearm crutches	AFO	35
S8	15	T1	D	W/C	NA	45
S9	11	C4	C	W/C	NA	17
S10	18	C6	D	Single point cane	NA	NT
S11	7	C6	C	W/C	NA	26
S12	13	T4	C	W/C	NA	20
S13	7	C6	D	Single point cane	AFO	39
S14	7	C3	D	Single point cane	AFO	49
S15	7	T7	D	Single point cane	NA	43
S16	12	T12	D	No assistive aid	NA	45
S17	12	C5	D	No assistive aid	NA	49

Abbreviations: W/C: using wheelchair for community ambulation; AFO=ankle foot orthosis; Bil=bilateral; LEMS: Lower extremity muscle score (normal is 50/50) as assessed by the American Spinal cord Injury Association (ASIA) motor impairment scale **(2001)**, NT = not tested, NA= Not applicable

Table 6-2. Percentage differences between the lower extremity maximum muscle CSA of wheelchair (W/C) and non-wheelchair (non-W/C) groups relative to corresponding controls. Data are expressed as differences in percentage means (% difference) ± standard error of percentage (s.e %).

		W/C group (n=7)	non-W/C group (n=10)
Muscle Groups	Individual muscles	% difference ± s.e%	
	SOL	27.5±7.5*	23.8±8.7†
Plantar flexors	MG	38.8±5.3*	13.7±11.8
	LG	30.1±9.8*	24.7±10.3†
Dorsi flexors	TA	20.7±6.1	26.1±8.3†
Knee extensors	QF	21.4±3.7* (n=6)	37.5±9.3† (n=8)
Knee flexors	HAMS	23.1±5.1 (n=6)	34.9±6.8† (n=8)

*Significant difference between the W/C and control groups ($p<0.05$). † Significant difference between the non-W/C and control groups. Abbreviations : SOL=soleus; MG=medial gastrocnemius; LG=lateral gastrocnemius; TA=tibialis anterior; QF = quadriceps femoris; HAMS=hamstrings

Table 6-3. Relative proportions of muscles in the pooled i-SCI and control groups.

Description	Proportions	Incomplete-SCI	Controls
Individual plantar flexors relative to posterior compartment	SOL:PC	0.47	0.47
	MG:PC	0.27	0.27
	LG:PC	0.16	0.17
Intra-compartment ratios‡	PF:TA	5.08	5.53
	QF:HAMS	1.94	1.99
Inter-compartment ratios []	QF:PF	1.74	1.70

SOL/PC = soleus/posterior compartment, MG/PC = medial gastrocnemius/posterior compartment, LG/PC = lateral gastrocnemius/posterior compartment; ‡Flexors and extensor ratios of leg and thigh: PF/TA= plantar flexor/Tibialis Anterior, QF/HAMS = Quadriceps/Hamstrings; []Proximal-distal antigravity muscle ratios: QF/PF = quadriceps/plantarflexor.

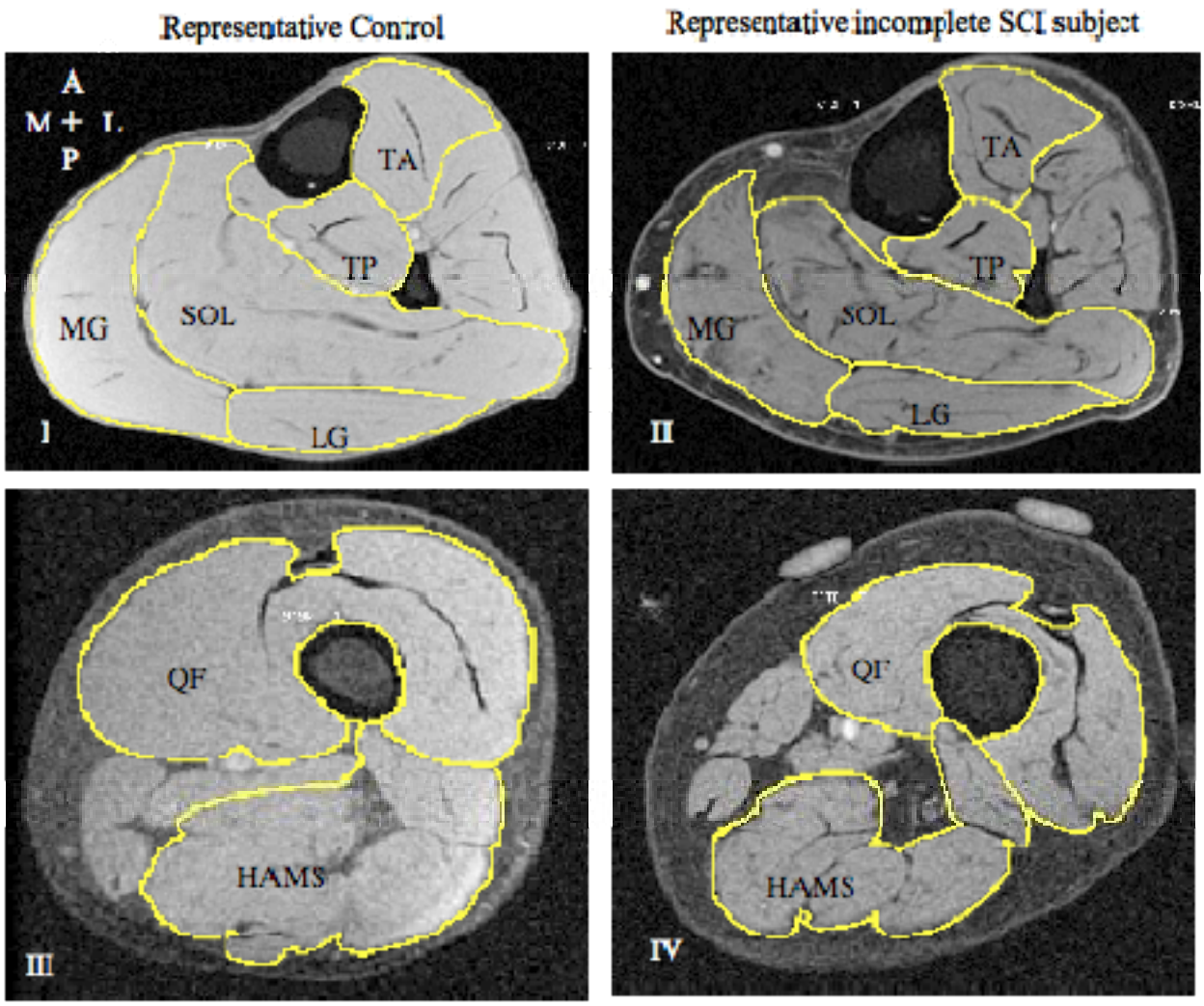

Figure 6-1. Representative trans-axial proton magnetic resonance images obtained at 1.5Tesla magnetic field. Leg (I, II) and thigh (III, IV) in a subject with incomplete-SCI (right) and corresponding age-matched control (left). [A= anterior; P = posterior; M=medial; L=lateral; MG=medial gastrocnemius; LG= lateral gastrocnemius; SOL=soleus, TP=tibialis posterior; TA=tibialis anterior, QF=quadriceps; HAMS=hamstrings].

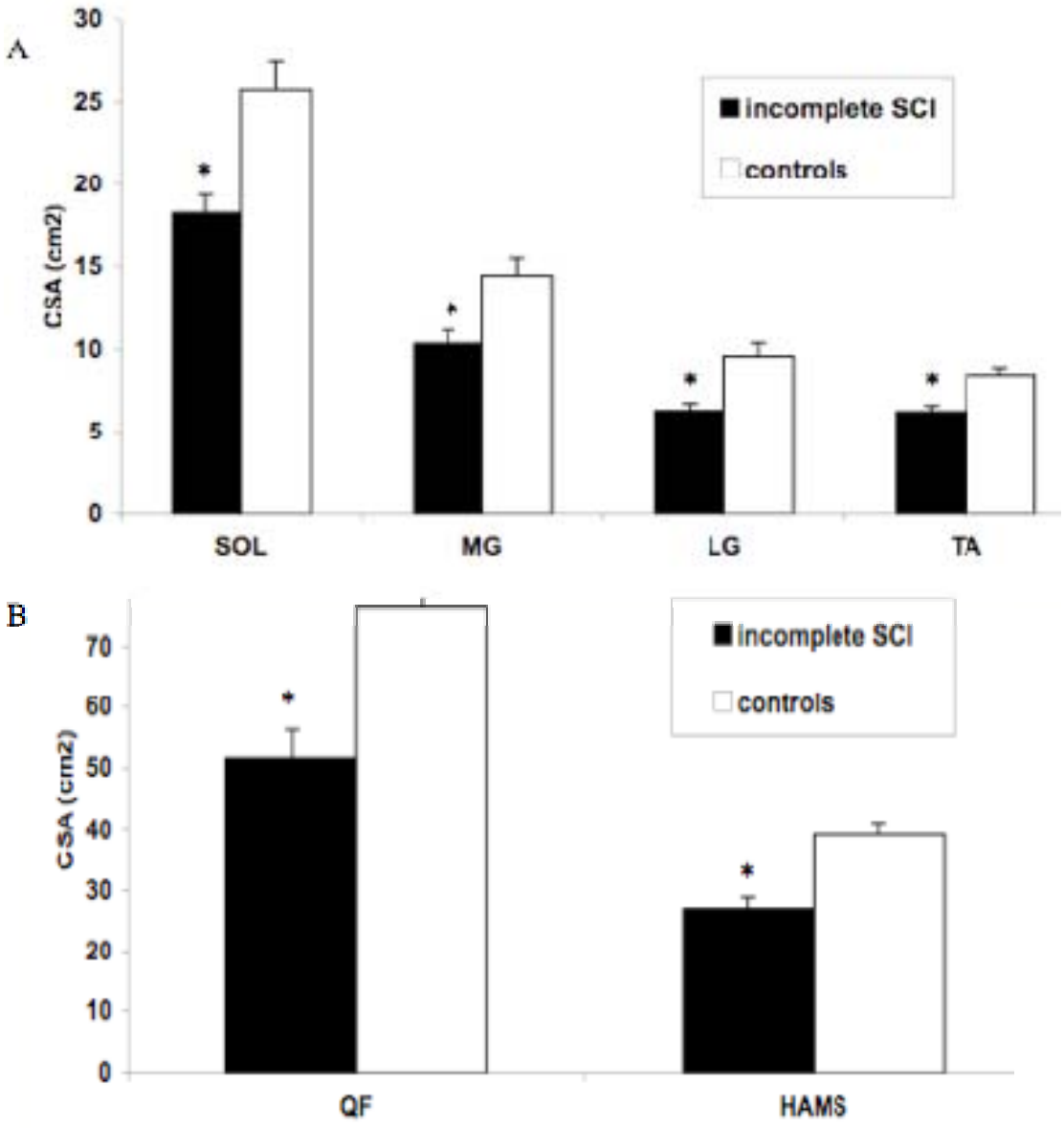

Figure 6-2. Muscle CSA in the pooled incomplete-SCI and control groups. A) leg muscles [SOL (soleus), MG (medial gastrocnemius), LG (lateral gastrocnemius) and TA (tibialis anterior)] and B) thigh muscles [QF (quadriceps) and HAMS (hamstrings)]. Values are means ± sem. * denotes significant differences between incomplete SCI and control subjects.

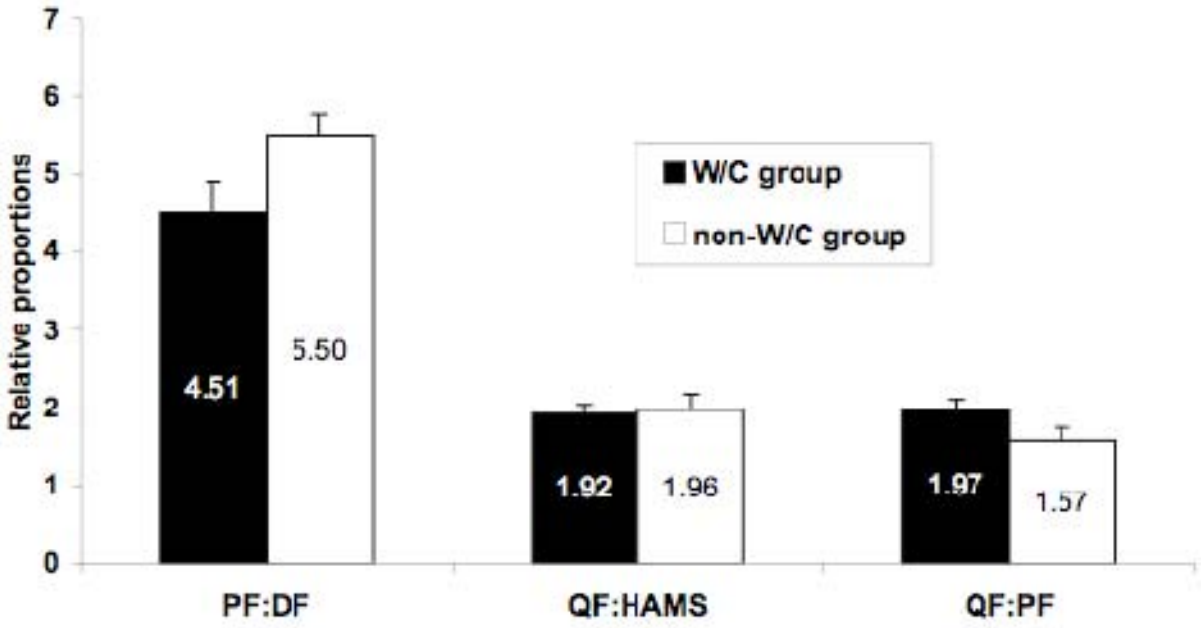

Figure 6-3. Proportion ratios of muscle groups within the leg and thigh. (PF/TA= plantar flexors/tibialis Anterior; QF/HAMS = quadriceps femoris/Hamstrings) and proximal and distal antigravity muscles (QF/PF = quadriceps femoris/plantar flexors) between the W/C and non-W/C group. * denotes significant differences between W/C and non-W/C incomplete SCI subjects.

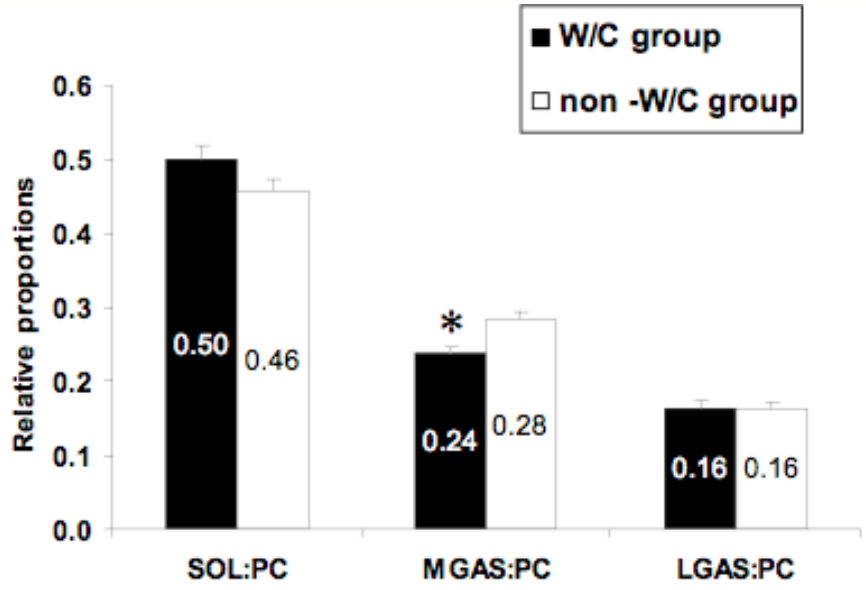

Figure 6-4. Ratio of individual plantar flexor muscles to the max CSA of the posterior compartment of the leg. (SOL/PC = soleus/posterior compartment, MG/PC = medial gastrocnemius/posterior compartment, LG/PC = lateral gastrocnemius/posterior compartment) in the W/C and the non-W/C group. * denotes significant differences between W/C and non-W/C incomplete SCI subjects.

CHAPTER 7
EXPERIMENT TWO – NON-INVASIVE ASSESSMENT OF LOWER EXTREMITY MUSCLE COMPOSITION AFTER INCOMPLETE SPINAL CORD INJURY

7.1 Summary

A sedentary lifestyle makes persons with SCI extremely vulnerable to cardiovascular complications. Glucose intolerance and insulin resistance is purported as one of the major metabolic risk factors in the development of cardiovascular complications after SCI. Several studies have established a positive correlation between the intramyocellular lipid (IMCL) concentration and insulin resistance in sedentary individuals and a variety of patient populations. Persons with complete spinal cord injury (SCI) show elevated intramuscular lipid levels that are well correlated with development of insulin resistance. The purpose of this study was to assess lower extremity muscle composition of persons with incomplete SCI. Combinations of T2 weighted magnetic resonance imaging from the calf muscles and localized unsuppressed proton spectroscopy from the soleus muscle were acquired in persons with chronic incomplete SCI and age-matched controls. Results of the study show elevated T2 relaxation times of the soleus and gastrocnemius muscle in persons with incomplete SCI. In addition, estimates of both the intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) content of the soleus muscle in persons with incomplete SCI were 3-5 times higher compared to that in controls. Higher IMCL concentrations in skeletal muscle may indicate that persons with incomplete SCI are predisposed to the development of peripheral insulin resistance and have a relatively greater risk to develop Type 2 diabetes.

7.2 Background

Amongst the 10,000 spinal cord injuries (SCI) that occur annually in the United States, almost 55% are classified as incomplete (NSCISC 2006). Similar to complete SCI (Castro, Apple et al. 1999; Shields 2002), individuals with incomplete SCI display a variety of skeletal

muscle adaptations including a decrease in muscle cross sectional area (Shah, Stevens et al. 2006), and decrement in voluntary force production and muscle activation (Jayaraman, Gregory et al. 2006). Collectively, these musculoskeletal deficits limit the overall functional capabilities of this patient population (Waters, Adkins et al. 1994; Ulkar, Yavuzer et al. 2003; Kim, Eng et al. 2004). Furthermore, a relatively sedentary lifestyle following the neurological injury makes persons with incomplete SCI extremely vulnerable to metabolic risk factors. Glucose intolerance and insulin resistance is purported as one of the major metabolic risk factors in the development of cardiovascular complications after SCI (Dallmeijer, van der Woude et al. 1999; Bravo, Guizar-Sahagun et al. 2004; Jacobs and Nash 2004; Manns, McCubbin et al. 2005).

Strong evidence in the literature suggests obvious positive correlations between intramyocellular lipid (IMCL) and insulin resistance in individuals with a relatively sedentary lifestyle including healthy individuals (Krssak, Falk Petersen et al. 1999; White, Ferguson et al. 2006), elderly (Cree, Newcomer et al. 2004) and in persons with obesity (Sinha, Dufour et al. 2002; Weiss and Caprio 2006). Accordingly, IMCL depots in skeletal muscle may provide an indirect measure of insulin resistance. Given that the higher reported incidence of insulin resistance in persons with incomplete SCI (Bauman, Spungen et al. 1999; Bauman and Spungen 2001) and the relatively inactive lifestyle (Subbarao 1991; Ditunno, Burns et al. 2005), we hypothesized that the IMCL content would be elevated in the lower extremity skeletal muscles of these individuals. Though studies have demonstrated elevated lipid depots in the lower extremity muscles of persons with complete and incomplete SCI using magnetic resonance imaging (Elder, Apple et al. 2004; Gorgey and Dudley 2006), we are not aware of any studies that have specifically reported alterations in the IMCL content in persons with motor incomplete SCI.

Therefore, the overall objective of this study was to investigate alterations in the skeletal muscle composition of persons with motor incomplete SCI using a combination of non-invasive magnetic resonance imaging (MRI) and spectroscopic (MRS) measures. Specifically, we measured the T_2 relaxation times of lower leg muscles and quantified the intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) content of the soleus muscle in persons with incomplete SCI.

7.3 Methods

General design: We performed a case-control study in which lower extremity muscle characteristics were compared between individuals with incomplete-SCI and age, gender, weight and height matched, non-injured healthy persons. For this purpose, we used a combination of proton imaging and spectroscopy techniques.

Subjects: Eight individuals (2 women) with chronic (17±9 months post injury) motor incomplete SCI (C4-T12; ASIA C or D) participated in this study. Four subjects used a cane or forearm crutches, while four used a powered wheelchair as their primary means of mobility (Table 7-1). In addition, eight able-bodied persons matched in age, weight, height and gender volunteered to serve as control subjects (mean ± SD; 42±11 yrs old, 73±13kg, 174±10cm). Control subjects were recreationally active, but not engaged in any rigorous exercise program, and were recruited from the Gainesville, FL community.

Prior to participating in the study, written informed consent was obtained from all subjects, as approved by the Institutional Review Board at the University of Florida, Gainesville. The conduct of all investigation conformed to the protocol and the ethical and humane principles of research.

Magnetic resonance measurements: A total of eight persons with incomplete SCI participated in the study. However, for logistical reasons, magnetic resonance imaging was

performed on seven individuals and proton magnetic resonance spectroscopy was performed on six participants.

Details of T2 weighted Magnetic Resonance Imaging (MRI section 5.1.4) and Proton Magnetic Resonance Spectroscopy (MRS section 5.1.5) measures and data analysis are provided in chapter 5.

Statistical analysis: Levene's test showed unequal variance between the SCI and control groups for both T_2 and lipid measures. Accordingly, non-parametric Mann Whitney tests were used to compare the T_2 relaxation times and baseline lipid measures between the SCI and control groups. SPSS for Windows (Version 11.0.1)[4] was utilized for all statistical analyses. Alpha level was set at 0.05.

7.4 Results

Muscle T2 relaxation times: Representative T_2 weighted axial images of the lower leg of healthy controls, ambulatory and non-ambulatory incomplete SCI subjects are provided in Figure 7-1. Overall, persons with incomplete SCI showed 11% -26% higher T_2 relaxation values in the tested lower leg muscles (Table 7-2). Significant differences were observed in the T_2 relaxation times of the SOL (p= 0. 011, 11%) and MG (p=0.005; 26%) muscles. Interestingly, all persons with incomplete SCI showed higher T_2 values of the MG and SOL muscles obtained by MRI. In contrast, the T_2 relaxation time of bone marrow was consistent in the SCI and control subjects (1% difference).

Calculations of soleus muscle T_2 relaxation times by spectroscopy coincided with T_2 measurements from MRI. Patients with an incomplete SCI showed a significant (14%, p=0.017) increase in the baseline T_2 relaxation times of the soleus muscle as compared to the control group [median (minimum-maximum) 35.8ms (30.8 – 41.5ms) versus 31.1ms (29.1-31.4ms)]

[4] SPSS Inc, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606

Intramuscular lipid: Persons with incomplete SCI demonstrated significantly higher total lipid, IMCL and EMCL levels in the soleus muscle as compared to healthy controls. Spectra acquired from the soleus muscle of healthy controls, ambulatory and non-ambulatory incomplete SCI subjects are provided in Figure 7-2. As shown in Figure 7-3, the total lipid content normalized to water was on average 3.2 times higher in the subjects with incomplete SCI as compared to controls. IMCL and EMCL values were 3.3 and 4.5 times higher in the incomplete SCI subjects. Note that incomplete SCI subjects also showed a much wider distribution of values for all lipid ratios as compared to the controls (Figure 7-3). Finally, individual data revealed that every person, ambulatory and non-ambulatory, with incomplete SCI showed an elevation in the overall lipid, IMCL and EMCL measures as compared to their corresponding controls (Figures 7-4 and 7-5).

7.5 Discussion

This study utilized T_2 weighted imaging and localized proton spectroscopy to characterize skeletal muscle in persons with chronic, motor incomplete SCI. Our results show marked elevations in the T_2 relaxation times of the locomotor muscles - soleus and medial gastrocnemius, as compared to controls, with maximum relative changes in the medial gastrocnemius muscle. In addition, estimates of both the intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) content of the soleus muscle in persons with incomplete SCI were 3-5 times higher compared to that in controls. Higher IMCL concentrations in skeletal muscle may indicate that persons with incomplete SCI are predisposed to the development of peripheral insulin resistance and have a relatively greater risk to develop Type 2 diabetes.

Using image-guided volume localized proton spectroscopy we quantified intramuscular lipid in the predominantly slow twitch soleus muscle. Interestingly, we found a marked increase in the intramuscular lipid content of all individuals with SCI compared to controls. This

phenomenon of elevated muscle lipid has been reported in a number of clinical populations including individuals with stroke (Ryan 2002) as well as both complete (Elder, Apple et al. 2004) and incomplete SCI (Gorgey and Dudley 2006). Elder et al demonstrated that persons with complete SCI display an almost 4-fold increase in intramuscular fat relative to controls, and that these values correlate with plasma glucose levels during an oral glucose tolerance test. In another study, a three fold elevation in intramuscular fat was observed in persons with chronic motor incomplete SCI (Gorgey and Dudley 2006). Previous studies in SCI patients however, did not distinguish between IMCL and EMCL, but instead used MRI to estimate total intramuscular lipid content. An advantage of the present study is that using localized H-spectroscopy we were able to individually quantify both IMCL and EMCL.

Our findings demonstrate significant elevations in the ratios of IMCL and EMCL to water following incomplete SCI. Moreover, the IMCL and EMCL content measured in the soleus muscle (relative to the water peak) of control subjects in our study fell within the range of similar measures reported in the literature (Krssak, Falk Petersen et al. 1999). Though IMCL is purported to be influenced by diet and exercise, studies also report strong correlations between IMCL and insulin resistance irrespective of diet, age, weight, activity level and gender (for reviews see (Goodpaster and Wolf 2004; Boesch, Machann et al. 2006). Nevertheless, in our study, while gender, body weight, physical activity and age were well controlled, elevations in myocellular lipids are far greater than those attributable to dietary influences (0.32 times elevation in previous dietary studies) (Stettler, Ith et al. 2005). The highly elevated IMCL levels in the incomplete SCI group raises concern because of their reported association with insulin resistance in sedentary individuals with normal body weights (Goodpaster and Wolf 2004; Boesch, Machann et al. 2006) as well as obese individuals (Visser, Kritchevsky et al. 2002;

Goodpaster and Wolf 2004). Moreover, skeletal muscle is the major depot (~80%) for blood glucose and marked muscle atrophy has been shown to be a secondary risk factor (Goodpaster and Kelley 1998; Goodpaster and Brown 2005). The combination of muscle atrophy (Shah, Stevens et al. 2006), relative inactivity (Subbarao 1991; Ditunno, Burns et al. 2005) and high IMCL levels in persons with incomplete SCI renders this patient population particularly vulnerable to altered glucose homeostasis.

In addition to measuring the intramuscular lipid content, we also studied the T2 relaxation characteristics of the lower extremity muscles in persons with chronic SCI. We found 11-26% higher T2 values in the lower extremity muscles of persons with incomplete SCI, with maximum relative changes in the medial gastrocnemius muscle. Because of partial volume filling estimates of muscle, T_2 relaxation time using MRI reflect properties of both water and lipid protons within the muscle. Lipid has an inherently longer T2 relaxation time (85-90ms) than muscle (T2 equal to 31-33ms) at 1.5T) (Bruhn, Frahm et al. 1991) and hence it is not surprising that persons with incomplete SCI, which show higher amounts of intramuscular lipid, would display increased T2 relaxation times on MRI. However, using proton spectroscopy we also assessed the T2 relaxation time of the soleus muscle independent of contributions of lipid, and found that the muscle T2 in persons with incomplete SCI remained elevated. Increased skeletal muscle T2 values have been reported in a number of conditions, including edema(Ploutz-Snyder, Nyren et al. 1997; Ababneh, Beloeil et al. 2005), peripheral denervation (Koltzenburg and Bendszus 2004; Wessig, Koltzenburg et al. 2004) and exercise induced muscle damage (Walter, Cordier et al. 2005).

Persons with motor incomplete SCI have vascular disturbances including venous vascular dysfunction (Hopman, Nommensen et al. 1994) and a continuous dependent position of their lower extremities may lead to accumulation of myocellular water in the postural leg muscles.

Increases in intramyocellular and extramyocellular water content can cause elevations in proton density and a subsequent increase in T2 relaxation times of muscle (Ploutz-Snyder, Nyren et al. 1997; Ababneh, Beloeil et al. 2005). Interestingly, we observed a pattern consistent with higher elevations in non-ambulatory subjects, that is, in persons who used a wheelchair for mobility (S5, S6, S7, S8). Furthermore, we found relatively greater T2 enhancements in the soleus and medial gastrocnemius as versus the tibialis anterior muscle. Concurrent to our findings, increases in muscle T2 relaxation times have also been reported after spinal contusion in rats. Liu et al have shown significant elevations in the T2 relaxation times of rat hind limb muscles after one week of spinal contusion (Liu, Bose et al. 2006). Interestingly, T2 elevations in the rat soleus muscle did not recover until three months after injury in contrast to the tibialis anterior muscle that showed normal T2 values by four weeks. The authors attributed the elevated T2 relaxation times to increase in extramyocellular fluid as indicated by an enhancement in extracellular space during concomitant qualitative histological assessment of the involved muscle (Liu, Bose et al. 2006). Similarly, elevated muscle T2 times after peripheral denervation in both humans (Uetani, Hayashi et al. 1993; Koltzenburg and Bendszus 2004) and animals (Wessig, Koltzenburg et al. 2004) have been attributed to capillary enlargement and increased muscular blood volume.

T_2 weighted MRI has also been successfully used to monitor muscle damage during reambulation following models of disuse such as cast immobilization (Frimel, Walter et al. 2005). Based on increase in muscle T2 after isometric contractions, previous studies suggest an increased susceptibility to muscle injury after complete spinal cord injury in humans (Bickel, Slade et al. 2004). Factors including unloading and inactivation of affected muscles, and skeletal muscle atrophy seemingly contribute to this phenomenon. Although persons with incomplete SCI have partial sparing of the spinal cord, relative unloading and inactivation of their lower

limb muscles might predispose skeletal muscle to injury. As such, the elevated baseline T_2 values from our data might be reflective of damaged muscle. However, we believe that muscle damage is a less likely factor in the present study because neither locomotor training in humans (submission under review) or animal (Liu, Bose et al. 2006) has shown to enhance muscle T2 after incomplete SCI. Using MR imaging and spectroscopy measures, we recently demonstrated that muscle loading and activation during locomotor training does not result in lower limb muscle damage after incomplete SCI in humans (submission under review). In fact, locomotor training in spinal contused rats has shown to accelerate the normalization in elevated muscle T2; with no link between muscle damage and T2 (Liu, Bose et al. 2006). Future studies using advanced imaging and spectroscopic measurements will be needed to identify the cause of elevated T_2 relaxation times.

We recognize potential limitations of our study. First, the sample size of our incomplete SCI group limits conclusions concerning the impact of injury level, duration and/or severity of injury on myocellular lipid levels. We observed a much larger variation in all lipid measures of our SCI group as compared to variation in a similar sized control group (see Figure 7-3). Since incomplete SCI reflects a very heterogeneous population with varying ambulatory status, future studies on a larger sample size with inclusion criteria based on severity of injury or duration after injury might prove worthwhile. Such studies can quantify skeletal muscle lipid composition, especially if these ratios could serve as non-invasive biomarkers of insulin resistance. Secondly, we did not test glucose tolerance or insulin sensitivity of our subjects; thereby limiting our ability to directly associate the myocellular lipids with the development of insulin resistance. Future studies on persons with incomplete SCI are warranted to confirm the association of IMCL accumulation and development of insulin resistance. Nevertheless, based on extensive literature

that correlates IMCL content with development of insulin resistance, we believe our data yield valuable information concerning potential risks associated with this type of injury and can provide a foundation for future studies.

In conclusion, we found an increase in T_2 relaxation properties of leg muscles and elevated fatty depots within the soleus muscle of persons with chronic incomplete SCI. Our results suggest altered muscle composition in this patient cohort. Future studies are warranted to identify the relationships between IMCL and insulin resistance along with the functional and physiological impacts of increased IMCL in this population.

Table 7-1. Characteristics of individuals with incomplete-SCI

Subject	Age (years) /Gender	BMI (kg/m2)	Months post injury	Injury level	ASIA Grade	Type of assistive aid	WISCI-II
S1	46/F	18.6	10	C5	D	Forearm crutch	15
S2	22/M	21.5	13	C5	D	NAD	20
S3	23/M	19.9	8	T1	D	Single point cane	19
S4	48/M	20.3	37	T1	D	Forearm crutch	12
S5	59/M	18.2	15	C6	C	Wheelchair	8
S6	42/F	28.3	16	C5	C	Wheelchair	6
S7	37/M	25.1	15	T1	D	Wheelchair	13
S8	58/M	27.4	11	C4	C	Wheelchair	8

Abbreviations: NAD= No assistive aid; WISCI-II = Walking Index for spinal cord injury (normal is 20/20).

Table 7-2. Percent differences between the T_2 relaxation times of lower extremity muscles in persons with incomplete SCI and corresponding controls (n=7). Data are expressed as median, minimum-maximum values (min-max) and percentage change in mean T_2 relaxation times.

	Incomplete SCI	Control subjects	% difference in T_2	p value
	median (min - max) of T_2 values by MRI			
SOL	31.7 (30.8 – 41.5)	30.5 (29.1 – 31.4)	11%	0.011*
MG	35.8 (30.1 – 45.7)	29.5 (27.8 – 32.4)	26%	0.009*
LG	33.0 (26.7 – 39.0)	30.9 (27.9 – 33.0)	11%	0.132
TA	28.8 (24.8 – 38.9)	27.0 (26.4 – 29.4)	12%	0.209
BM	49.2 (46.8 – 51.6)	47.9 (45.7 – 51.6)	1%	0.667

*Significant T_2 differences between the incomplete SCI and control groups ($p<0.05$). Abbreviations: SOL=soleus; MG=medial gastrocnemius; LG=lateral gastrocnemius; TA=tibialis anterior; BM = Bone marrow.

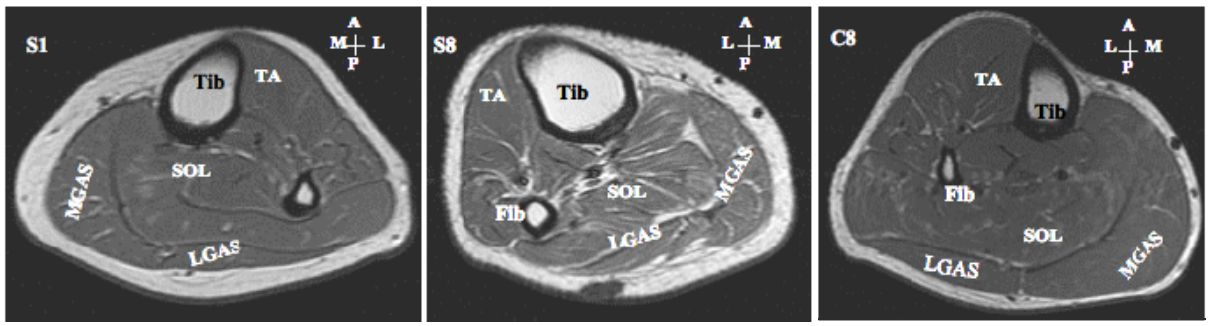

Figure 7-1. Representative T2 weighted trans-axial proton magnetic resonance images of the lower leg. Ambulatory individual with incomplete SCI (S1) ; non-ambulatory individual with incomplete SCI (S8) ; able-bodied control subject (C8). [A= anterior; P = posterior; M=medial; L=lateral; MG=medial gastrocnemius; LG= lateral gastrocnemius; SOL=soleus, TA=tibialis anterior]

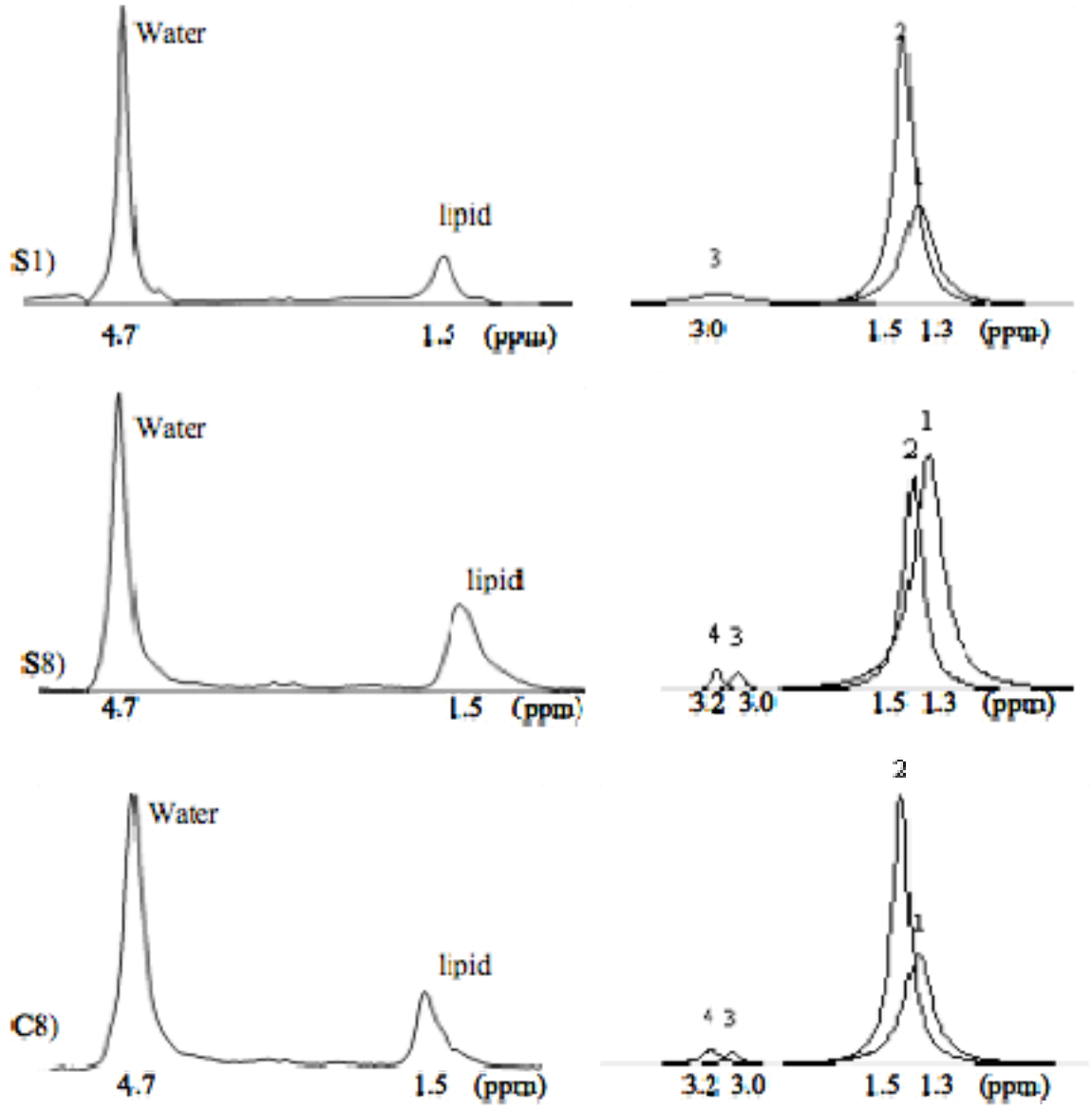

Figure 7- 2. Representative proton magnetic resonance spectra obtained from the soleus muscle. Ambulatory individual with incomplete SCI (S1); non-ambulatory individuals with incomplete SCI (S8) ; able-bodied control subject (C8). The left column represents proton spectra showing water (4.7ppm) and lipid (1.5ppm) peak. The right column illustrates lipid components after water suppression [1= intramyocellular lipid (IMCL) at 1.3ppm; 2 = extramyocellular lipid (EMCL) at 1.5ppm; 3 = creatine (CH_3) at 3.0ppm; 4 = choline at 3.2ppm]

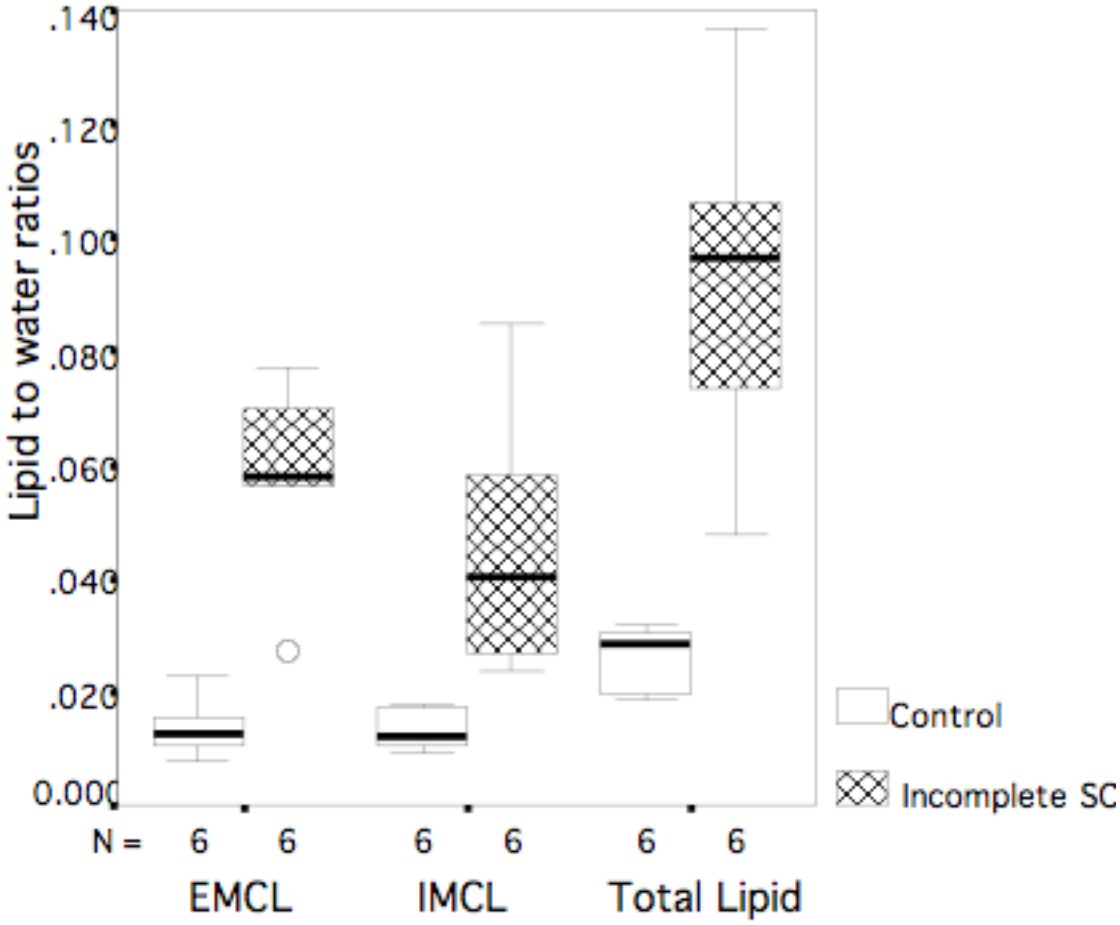

Figure 7-3. Box-plot depicting variability in EMCL/water, IMCL/water and total soleus muscle lipid/water ratios. Note the large variability in the SCI (hatched bars) versus control group for all ratios. The EMCL/water ratio shows an extreme value from subject S6 (O).

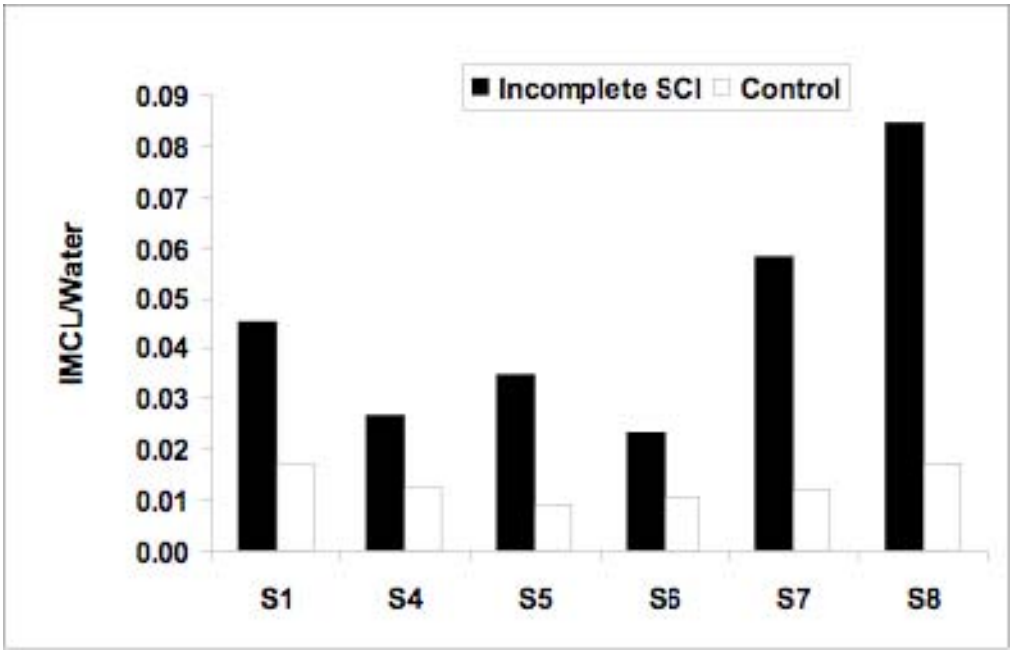

Figure 7-4. Individual data comparisons of IMCL to water ratio (IMCL/water)

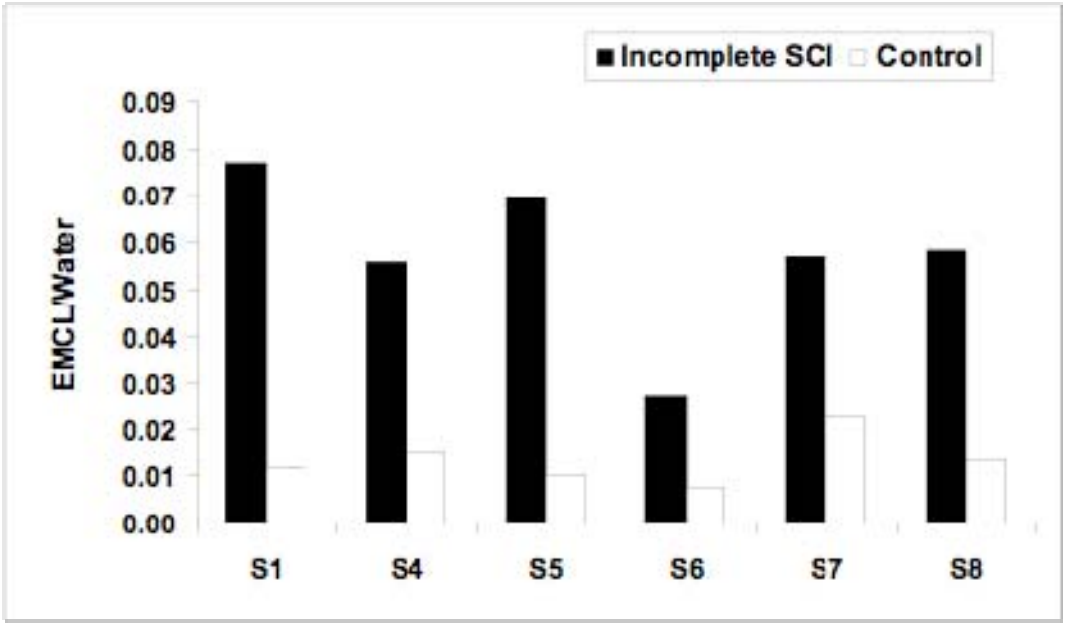

Figure 7-5. Individual data comparisons of EMCL to water ratio (EMCL/water)

CHAPTER 8
EXPERIMENT THREE - MAGNETIC RESONANCE ASSESSMENT OF MUSCLE DAMAGE DURING LOCOMOTOR TRAINING IN PERSONS WITH INCOMPLETE SPINAL CORD INJURY

8.1 Summary

The purpose of this study was to assess the impact of locomotor training (LT) on muscle T2, an in vivo marker of muscle damage before, at 2 weeks and 9 weeks of training in persons with incomplete spinal cord injury (SCI). Persons with chronic incomplete SCI underwent nine weeks of LT (45 sessions) that involved step training (30 minutes) on the treadmill with body weight support (BWS) and manual assistance followed by over ground walking (20 minutes). T2 relaxation time was measured in lower extremity muscles [soleus, medial gastrocnemius, lateral gastrocnemius and tibialis anterior] before LT and after 2 and 9 weeks of LT using Magnetic Resonance Imaging (MRI) and Spectroscopy (MRS). Overall, no significant differences were detected in the T2 relaxation times of lower extremity muscles of individuals with chronic i-SCI during LT using either MRI or MRS measures. Our pilot data suggest that muscle loading and activation during LT as provided in the present study does not result in significant muscle damage in the lower limb, following i-SCI as determined by MRI and MRS.

8.2 Introduction

Secondary to chronic alterations in loading and inactivity, persons with complete spinal cord injury experience extensive muscle atrophy (45-80%) and an increased susceptibility to muscle damage (Bickel, Slade et al. 2004). With recent advances in the management of acute SCI, most injuries are now classified as incomplete (i-SCI). Individuals with i-SCI demonstrate a 24-31% decrease in lower extremity muscle size, considerable loss of muscle strength and an increased dependence on the use of assistive devices for ambulation (Melis, Torres-Moreno et al. 1999; Shah, Stevens et al. 2006). However, the degree of functional impairment and reduction in

loading/activity after i-SCI is variable and the muscle's susceptibility to damage has not been studied.

Recently, rehabilitation of persons with i-SCI has focused on the use of treadmill locomotor training (LT) to facilitate recovery of walking (Wernig, Nanassy et al. 1998; Behrman, Lawless-Dixon et al. 2005). LT takes advantage of the phasic, peripheral sensory information and loading associated with stepping to promote neural plasticity and maximize residual function. However, a variety of animal studies have shown that reambulation following a period of unloading and inactivity can cause muscle damage in postural muscles such as the soleus (Kasper, White et al. 1990; Frimel, Walter et al. 2005). Consequently, the potential for muscle damage to occur during LT especially at the onset of training is a concern. Therefore the objective of this study was to determine the effect of two (2wk-LT) and nine weeks of LT (9wk-LT) on in-vivo markers of muscle damage in the lower limb muscles of persons with chronic i-SCI using magnetic resonance techniques.

8.3 Methods

Subjects: Seven subjects (48±9 yr; 70±14 kg; 175±13 cm) with chronic (17±10 months) motor i-SCI (C4-T12; ASIA C or D) participated in this study. Three subjects used a cane or forearm crutches, while four used a powered wheelchair as their primary means of mobility.

Locomotor training: LT consisted of 9 weeks (45 sessions) of step training (30 minutes) on the treadmill with body weight support and manual assistance followed by over ground training (20 minutes). A detailed description of the training principles, parameters and progression has been described in Chapter 5 (5.1.3) of this work.

Magnetic Resonance Imaging (MRI) and Spectroscopy (MRS): Details of T2 weighted Magnetic Resonance Imaging (MRI – section 5.1.4) and Proton Magnetic Resonance

Spectroscopy (MRS – section 5.1.5) to measures T2 relaxation times of muscles and data analysis are provided in chapter 5.

Statistical analysis: A two-way repeated measures ANOVA was used to test for differences in T2 relaxation times. SPSS for Windows (v11.0.1)[5] was utilized for all statistical analyses ($\alpha = 0.05$).

8.4 Results

No significant differences were found in the T2 relaxation times of lower limb muscles in persons with chronic i-SCI following LT using either MRI or MRS. Muscle T2 relaxation times measured using MRI and MRS in the tested muscles are provided in Table 8-1. The T2 relaxation times of bone marrow did not change (variance = 4%) with LT.

8.5 Discussion

Pilot data presented in this study demonstrate that during 2 weeks and 9 weeks of LT, T2 relaxation times of lower extremity muscles do not significantly change in persons with chronic i-SCI. These results indicate that body weight supported LT does not induce muscle damage in affected lower extremity muscles of persons with chronic i-SCI, despite significant muscle atrophy and weakness.

Several studies have demonstrated that chronic unloading and inactivity renders skeletal muscles more susceptible to muscle damage. Using MRI, Bickel et demonstrated that persons with complete SCI experience muscle damage even after a single bout of isometric contractions (Bickel, Slade et al. 2004). Alterations in the MR T2 relaxation properties of skeletal muscle, indicative of muscle damage, have also been reported during reambulation following cast immobilization (Frimel, Walter et al. 2005). MRI has been used extensively to monitor muscle damage in a variety of conditions. It has the distinct advantage to provide spatial information and

[5] SPSS Inc, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606

allows the investigation of multiple muscles simultaneously. However, similar to other imaging techniques, T2 weighted MRI suffers from partial volume filling, making it challenging to reliably assess the inherent T2 of skeletal muscle in the presence of increased amounts of intramuscular lipid. MRS on the other hand, while providing limited spatial information, offers the opportunity to monitor changes in muscle T2 without contamination from lipid signal (Walter, Cordier et al. 2005). In this study, we found no significant change in the T2 relaxation times of lower extremity muscles during LT using either method.

Of note, our data demonstrate relatively higher baseline (pre-LT) T2 values in the i-SCI group relative to published control values at the same magnetic field strength (Akima, Ushiyama et al. 2003). Seemingly, some baseline alterations in muscle composition may be present in skeletal muscle of persons with i-SCI, independent of ambulatory status, which warrants further investigation.

8.6 Conclusion

In conclusion, these pilot data suggest that bodyweight supported treadmill LT does not induce muscle damage in the lower-limb muscles of persons with chronic i-SCI.

Table 8-I. MR measures of T2 relaxation times of lower extremity muscles before and after LT. The soleus (SOL), medial gastrocnemius (MGAS) and lateral gastrocnemius (LGAS) and tibialis anterior (TA) muscles prior to LT (pre-LT), after two weeks (2wk-LT) and nine weeks (9wk-LT) of locomotor training in persons with incomplete SCI.

T_2 Relaxation times	Muscle	Pre-LT	2wk-LT	9wk-LT
		[Mean T_2 (ms) ± standard error]		
Magnetic Resonance Imaging	SOL	33.7±1.5	37.1±1.9	36.0±2.0
	MGAS	36.9±2.7	38.8±2.4	38.8±2.6
	LGAS	33.5±1.8	37.2±3.2	38.4±3.3
	TA	30.9±1.9	33.5±1.4	32.6±1.9
	Bone marrow	49.7±0.6	49.9±0.5	50.1±0.3
Magnetic Resonance Spectroscopy	SOL	34.5±1.5	37.1±0.7	37.2±1.2

CHAPTER 9
EXPERIMENT FOUR - IMPACT OF LOCOMOTOR TRAINING ON MUSCLE SIZE AND INTRAMUSCULAR FAT AFTER SPINAL CORD INJURY

9.1 Summary

The use of repetitive locomotor training with body weight support to improve motor recovery and ambulation following incomplete-SCI has gained considerable momentum in the last two decades. The purpose of the present study was to investigate if LT impacted muscle physiology; especially lower extremity whole muscle size and fat content in persons with incomplete SCI. Nine persons with incomplete SCI underwent 45 sessions of locomotor training protocol that consisted of treadmill stepping and over ground walking (LT). Magnetic resonance imaging and proton spectroscopic measures were utilized to measure lower extremity muscle size and soleus muscle composition after nine weeks of the training. LT effect on skeletal muscle was variable. Our findings show significant increases (8-10%) in the plantarflexor muscle cross sectional area of persons with incomplete SCI after the LT; with no marked changes in the thigh muscles. In addition, LT did not cause any alterations in the soleus muscle lipid composition in our patient cohort.

9.2 Introduction

Atrophy of paralyzed muscles is one of the most obvious muscular adaptations following SCI. Depending upon the severity of injury; individuals with SCI demonstrate a noticeable 25-80% decline in average cross-sectional area (CSA) of their lower leg and thigh muscles (Castro, Apple et al. 1999; Shah, Stevens et al. 2006). Consequent to this atrophy are a battery of disabling motor impairments including decreases in absolute muscle force production, muscle torque and power (Lieber 1986; Shah, Stevens et al. 2006). Functionally, atrophy significantly diminishes muscle strength, motor performance, and locomotor capabilities and makes the paralyzed muscle susceptible to injury (Bickel, Slade et al. 2004; van Hedel, Wirth et al. 2005).

Recently, atrophy following SCI has also been linked to substitution of the paralyzed muscle by fatty tissue infiltration (Elder, Apple et al. 2004; Gorgey and Dudley 2006). Though direct functional implications of the presence of fat in skeletal muscle remain unidentified at this time, wide arrays of studies have reported strong associations between intramuscular fat, specifically intramyocellular lipid (IMCL) and development of insulin resistance (Perseghin, Scifo et al. 1999; Furler, Poynten et al. 2001). Furthermore, persons with SCI are predisposed to development of insulin resistance (Bauman, Spungen et al. 1999; Bauman and Spungen 2001) that shows a significant association with muscle fat (Elder, Apple et al. 2004).

Several studies have aimed at attenuating atrophy after SCI to optimize limb function and improve the locomotor capabilities of this patient cohort (Bremner, Sloan et al. 1992; Dudley, Castro et al. 1999; Creasey, Ho et al. 2004). An emerging trend in promoting motor recovery and ambulation following incomplete-SCI is the use of repetitive locomotor training with body weight support (Edgerton, Tillakaratne et al. 2004; Behrman, Bowden et al. 2006). Locomotor training has been suggested to have a positive impact on the walking ability, functional independence and subjective well being of persons with chronic incomplete SCI (Phillips, Stewart et al. 2004; Behrman, Bowden et al. 2006; Hannold, Young et al. 2006). Moreover, studies have shown that locomotor training involves sufficient mechanical loading to increase muscle fiber size and muscle glucose tolerance, while simultaneously enhancing locomotor capacities (Phillips, Stewart et al. 2004; Stewart, Tarnopolsky et al. 2004; Adams, Ditor et al. 2006). However, to our knowledge, no study has investigated the effects of locomotor training on lower extremity whole muscle size and fat content of skeletal muscle.

Therefore, the main purpose of this study was to assess the effect of locomotor training on the muscle size and fat content of lower extremity muscles after incomplete SCI. Specifically,

we measured the cross-sectional area of the lower leg and thigh muscles and quantified fat content (along with intramyocellular lipid and extramyocellular lipid) of the soleus muscle after 9 weeks of locomotor training in persons with incomplete SCI.

9.3 Methods

Subjects: Nine individuals (2 women) with chronic (17±9 months post injury) motor incomplete SCI participated in this study. Details of subject selection are provided in Chapter 5. Five subjects used a cane or forearm crutches, while four used a powered wheelchair as their primary means of mobility.

Locomotor training protocol: Described in chapter 5

Magnetic resonance measurements: For logistical reasons, magnetic resonance imaging for cross sectional area measurements was performed on nine individuals and proton magnetic resonance spectroscopy for estimation of lipid content was performed on five participants. Details of the procedures and data analysis are provided in chapter 5.

Statistical analysis: Paired t-tests were used to compare CSA of the plantar flexors and thigh muscles before and after nine weeks of LT. In addition, non-parametric Mann Whitney tests were used to determine the effect of LT on TA muscle CSA and the lipid content. SPSS for Windows (Version 11.0.1)[6] was utilized for all statistical analyses. Alpha level was set at 0.05.

9.4 Results

Nine weeks of LT produced selective hypertrophy of lower extremity muscles in persons with incomplete SCI (Figures 9-1). Significant changes were found in the CSA of the individual plantarflexor muscles after nine weeks of LT. SOL muscle CSA increased by 8% ($p=0.040$) and

[6] SPSS Inc, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606

the MG (p=0.046) and LG (p=0.045) increased by 10%. Increases in CSA of the TA (4%), QF (4%) and HAMS (6%) were not significant after LT (Figure 9-2).

No effect of LT was seen in the total lipid, IMCL and EMCL contents of the soleus muscle (Figure 9-3).

9.5 Discussion

Findings of our present study reveal selective hypertrophy of the plantar flexors and no change in the dorsiflexors (TA) and thigh muscle size after nine weeks of LT in persons with incomplete SCI. In addition, LT does not alter the lipid content of the soleus muscle in our patient group.

Our results are in concurrence with animal models of spinal transection, where maximum effect of LT is seen in the slow extensor muscles with minimal or no effect on the fast extensor or flexor muscles (Roy and Acosta 1986; Roy, Talmadge et al. 1998). In addition, a 23% increase in the soleus muscle fiber cross-sectional area has been shown after one week of locomotor training in spinal contused rats (Stevens, Liu et al. 2006). To our knowledge, no studies in humans have determined the impact of LT on whole skeletal muscle size. Previous studies in humans have shown that around 65 sessions of locomotor training with manual assistance from therapists increases vastus lateralis mean fiber area by almost 25% in persons with incomplete SCI (Stewart, Tarnopolsky et al. 2004). While our study demonstrates significant hypertrophy of the plantarflexor muscles, we see an insignificant 4% increase in the overall size of the quadriceps muscles after LT. In the study by Stewart et al, the greater number of training sessions might have probably produced the quadriceps hypertrophic response. In contrast to LT, comparatively lesser training frequencies (as less as 30 sessions) of strength training interventions such as electrical stimulation has shown relatively greater hypertrophy of

the treated musculature. In persons with incomplete SCI, Gregory et al have reported an increase in quadriceps muscle CSA by 8% and of the plantar flexors by 14% after 30 sessions of electrical muscle stimulation accompanied with plyometric training (Gregory, Bowden et al. 2007). Sloan et al have shown an increase in the quadriceps muscle CSA of chronic incomplete SCI individuals by 9% after similar sessions of electrical stimulation induced cycling training (Sloan, Bremner et al. 1994). These morphological improvements in muscle size have also been associated with simultaneous gains in muscle strength and subjective reports of functional activities of daily living. Despite relatively lesser training sessions, hypertrophy observed in these studies is probably because of the hypertrophic stimulus of strength training that is achieved because of specific muscle activity against adequate resistance. In contrast, the intensity of LT used at every session in the current study was just enough to generate an alternating slow stepping pattern that simulates near-normal walking. Though subjects in our study gradually progressed to lesser body weight support, the speeds and overall duration of training might have deemed insufficient to elicit a major hypertrophic response. Perhaps, a much stronger stimulus is necessitated to elicit specific quadriceps hypertrophy. On the other hand, the gain in CSA of the plantarflexor muscles after LT is probably because of their selective activation during the training. LT protocol in our study placed constant emphasis on adequate foot placement and push off that largely involves activity in the plantar flexor muscles. Deitz et al show have shown that four-five weeks of LT drastically increases the EMG activity of gastrocnemius muscle in individuals with incomplete SCI as compared to the dorsiflexors or thigh muscles (Dietz, Colombo et al. 1995). Of note, our sample size (n=6) for the thigh muscle CSA data limits us from making any conclusive remarks about LT effect on thigh muscle size. Nevertheless, our findings suggest that LT has the potential to selectively enhance skeletal muscle size.

In response to LT in our patient group, we did not find any significant alterations in the soleus muscle fat content after 9 weeks. The phenomenon of elevated muscle lipid has been reported in a number of clinical populations including individuals with stroke (Ryan 2002), multiple sclerosis (White 2007) as well as both complete (Elder, Apple et al. 2004) and incomplete SCI (Gorgey and Dudley 2006). A common precipitating factor for increased fatty tissue infiltration in these conditions is the presence of a sedentary lifestyle. Evidence from literature demonstrates an improvement in glucose uptake in skeletal muscle (hence a decrease in insulin resistance) after LT in persons with incomplete SCI (Phillips, Stewart et al. 2004). Given the strong associations of IMCL with insulin resistance (Jacob, Machann et al. 1999; Krssak, Falk Petersen et al. 1999; Schrauwen-Hinderling, Hesselink et al. 2006), we hypothesized that increased physical activity through LT would decrease the accumulated muscle fat (and hence IMCL) in our patient cohort. Using non-invasive methods of MRS we have been able to quantify the lipid components in the soleus muscle. However, we did not encounter any alterations in the lipid content in our present study. Counterintuitive as it might seem, but studies have unequivocally shown an increase in the IMCL content after both short and long term endurance training (such as running and cycling) in healthy individuals (VanLoon 2004). Apparently, despite an increase in the IMCL content, endurance trained individuals (specifically athletes), have markedly greater insulin sensitivity. Studies suggest that unlike in sedentary individuals, excess IMCL in endurance athletes actually contributes positively by playing an integral role in mitochondrial oxidation and increasing the oxidative capacity of the muscle (Schrauwen-Hinderling, Hesselink et al. 2006). We are unsure however, if the intensity of LT used in this study makes a difference in altering muscle lipid content. Phillip et al have shown an increase in the glucose uptake after LT in persons with chronic incomplete SCI; thereby suggesting an

increase in sensitivity to insulin resistance in this patient cohort. However, patients in their study underwent 68 sessions of LT as versus 45 sessions in the present study. Nevertheless, based on our sample size and non-availability of direct measures of insulin resistance, interpretations on insulin sensitivity and any potential of relationship of IMCL with oxidative capacity etc are difficult to make.

As a potential limitation, the sample size of our incomplete SCI group limits conclusions concerning the impact of high intensity of LT on atrophy and myocellular lipid levels. Furthermore, we simply studied the soleus muscle to assess lipid composition and different muscles might respond to therapy differently. Future studies are probably needed to identify effect of different doses (duration and intensity) and types of exercise (endurance versus strength training on atrophy and lipid content in persons with incomplete SCI. Concurrent measurements of oxidative capacity and standard measurements of insulin sensitivity will help verify the direct effects of these trainings on the paralyzed skeletal muscle.

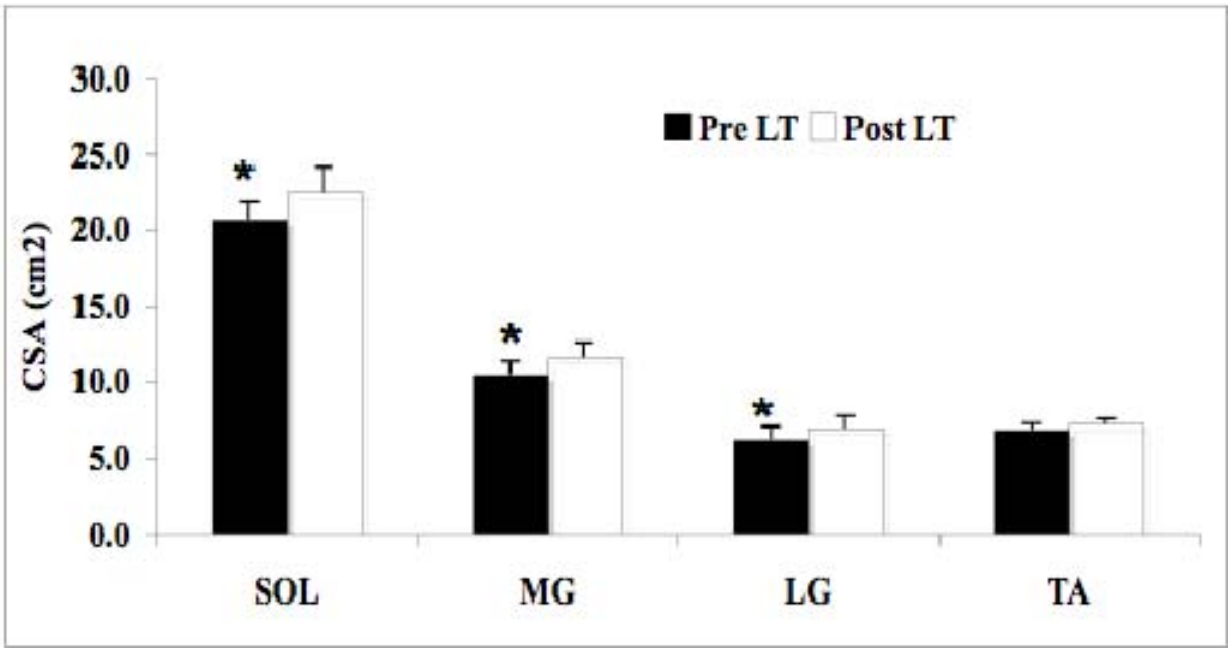

Figure 9-1. Cross sectional area of lower leg muscles after nine weeks of locomotor training (9wk-LT). *Significant change in selective plantar-flexor muscles after LT).

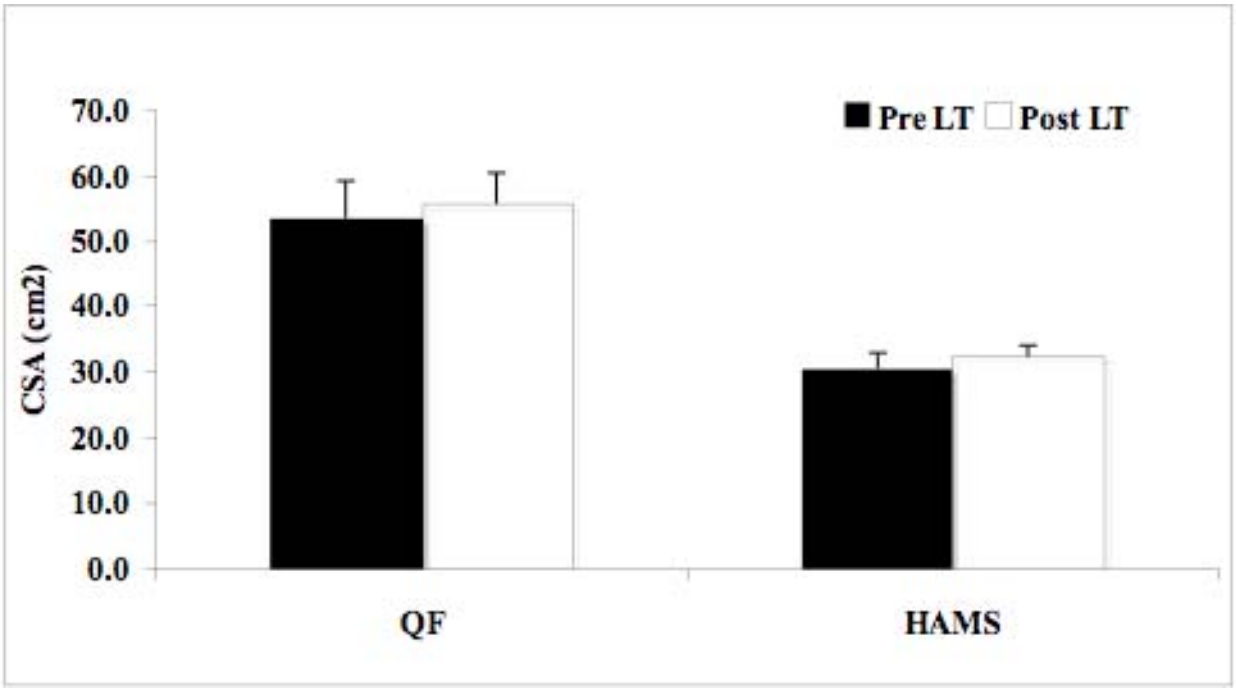

Figure 9-2. Cross sectional area of thigh muscles after nine weeks of locomotor training (9wk-LT). LT did not change CSA of the thigh muscles in persons with incomplete SCI.

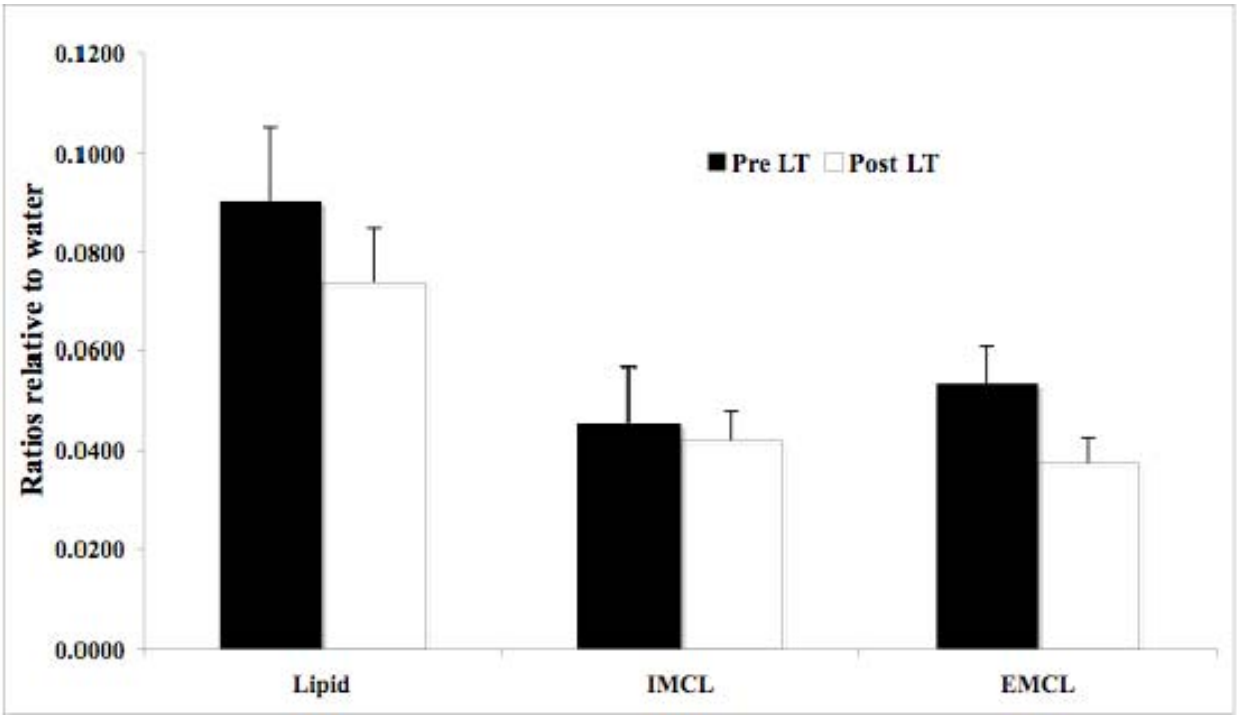

Figure 9-3. Estimates of soleus muscle lipid before (Pre LT) and after (Post LT) nine weeks of locomotor training (LT) in persons with incomplete SCI. LT did not produce any significant change in muscle lipid composition.

CHAPTER 10
EXPERIMENT FIVE - MONITORING ALTERATIONS IN INORGANIC PHOSPHATE OF HINDLIMB MUSCLE AFTER SPINAL CORD CONTUSION IN RATS

10.1 Summary

The overall objective of the present study was to determine the inorganic phosphate content [Pi] and phosphorylation potential of the rat hindlimb muscle after moderate spinal cord contusion in rats. Eight young adult female rats were moderately injured at the T8-T10 thoracic spinal cord. ^{31}P MRS measurements were performed at weekly intervals for assessments of phosphorylation potential of the rat hindlimb muscle for three weeks. Spectra were acquired in a Bruker 11T/470 MHz spectrometer using a ^{31}P (190.5 MHz) surface coil, placed over the belly of the gastrocnemius muscles. Resting spectra were manually phased, and the areas of the γ-ATP, Pi, and PCr peaks were determined using area integration. Absolute concentrations of the metabolite ratio were obtained after accounting for correction factors and through biochemical determination of total creatine [TCr] and [ATP] content in gastrocnemius muscle. Our data revealed marginally significant elevation in [Pi]/[PCr] ratios, significantly elevated [ADP][Pi]/[ATP] ratios and marked decreases in [PCr]. These data suggests an imbalance of the intracellular energy buffer system of the paralyzed hindlimb muscle that recovers by three weeks after injury.

10.2 Introduction

Relative inactivity and a sedentary lifestyle following incomplete SCI leads to unloading and disuse of the paralyzed lower extremity muscles (Melis, Torres-Moreno et al. 1999). We recently reported marked skeletal muscle atrophy and an overall decrease in the ability of lower extremity skeletal muscles to generate muscle peak torque in persons with incomplete SCI (Shah, Stevens et al. 2006).

Studies of muscle disuse show evidence that forced inactivity of lower extremity muscles (i.e. immobilization, limb suspension and denervation) result in metabolic alterations of involved musculature that may ultimately hinder muscle function (Lai, Jaweed et al. 1992; Vandenborne, Elliott et al. 1998; Yoshida, Ikata et al. 2001; Pathare, Walter et al. 2005). Specifically, studies have shown that cast immobilization is accompanied by marked elevations in the basal inorganic phosphate (Pi) concentration and the Pi-to-PCr ratio. Pathare et al demonstrated elevated levels of resting Pi in the immobilized skeletal muscles of patients with an orthopedic injury (Pathare, Walter et al. 2005) and also in a mouse model of cast immobilization (Pathare, Vandenborne et al. 2007). Pi/PCr ratio is closely related to the phosphorylation potential and reflects the energy state of the muscle (Veech, Lawson et al. 1979; Chance 1984). Though the exact mechanisms of elevated Pi levels secondary to disuse are not well established, pathophysiological processes accompanying disuse itself have been hypothesized as potential contributions (Lai, Jaweed et al. 1992; Pathare, Vandenborne et al. 2007). Notably, elevated Pi levels in the above-mentioned studies have been shown to correlate well with declines in muscle force. In-vitro skinned muscle fiber studies have unanimously reported that inorganic phosphate suppresses skeletal muscle force production (Kentish 1986; Chase and Kushmerick 1988; Martyn and Gordon 1992). Inhibitory effects of Pi on force development are shown to result from alterations in Ca^{+2} sensitivity of myofibrils that subsequently impacts the actomyosin cross-bridge force-Ca^{+2} relationship (Kentish 1986).

Increases in basal Pi concentration have also been noted following peripheral denervation. Lai et al showed that following crush denervation of the sciatic nerve, the Pi/PCr ratios increased in the denervated gastrocsoleus muscle (Lai, Jaweed et al. 1992). These ratios returned to normal values following reinnervation. Interestingly, metabolic recovery of the muscle showed similar

patterns of recovery as parameters of nerve conduction (Lai, Jaweed et al. 1992). In addition, spinal transection studies in the animal model have shown tendencies of an altered phosphorylation potential after SCI (Durozard, Gabrielle et al. 2000). However, these studies on metabolic adaptations following neurological injuries have been conducted at relatively lower magnetic field strengths and suffer from low signal to noise ratios.

The main aim of the present study was to characterize metabolic adaptations; specifically the quantification of phosphate metabolites (and hence the phosphorylation potential) of the resting rat hind limb muscle following spinal cord contusion. We used non-invasive phosphorus magnetic resonance spectroscopy (^{31}P- MRS) at magnetic field strengths of 11Tesla to monitor the phosphate metabolite content in the contused rat hind limb muscles over a course of three weeks.

10.3 Specific Aims and Hypothesis

10.3.1 Specific Aim

a) To determine the impact of acute spinal contusion (one week) on the resting phosphate metabolite content, and hence the phosphorylation potential of rat calf muscle after spinal cord contusion. b) To longitudinally monitor alterations in the phosphorylation potential of the calf muscles.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) at a high magnetic field strength (11T) was used to monitor basal phosphate metabolites for three weeks after spinal cord contusion. Skeletal muscle ATP and total creatine content (TCr) were quantified by biochemical assays.

10.3.2 Hypotheses

a) Immediately after spinal cord contusion (one week), there is an alteration in the resting phosphate metabolite content and hence a change in the phosphorylation potential of the

paralyzed hind limb muscle. b) Muscle phosphate metabolites begin to recover at three weeks after spinal cord contusion.

10.4 Methods

10.4.1 Experimental Design

Two groups of randomly distributed rats were studied. Animals either underwent a spinal cord contusion (n=8) or served as controls for wet lab procedures relevant to the study (see below). The spinal injured group underwent ^{31}P magnetic resonance spectroscopic (^{31}P-MRS) measurements prior to injury and at weekly intervals for three weeks after injury.

10.4.2 Animals

Sixteen adult Sprague Dawley female rats (12 week, 228-260g; Charles River, NJ) were housed in a temperature controlled room at 21^{0}C with a 12:12 hours light: dark cycle and provided with rodent chow and water *ad libitum*. Of these, 8 rats were moderately injured at the T8-T10 thoracic spinal cord levels and 8 rats served as controls in providing healthy muscle tissue to run biochemical assays for the quantification of phosphate compounds. The control group also provided reproducibility results of our ^{31}P data. All experimental procedures were performed in accordance with the U.S. Government Principle for the Utilization and Care of Vertebrate Animals by approval of the Institutional Animal Care & Use Committee at the University of Florida.

Details about spinal cord contusion operation procedures have been described in detail in chapter 5 (Section 5.2.2)

10.4.3 Magnetic Resonance Spectroscopy: Data Collection and Analysis

Phosphate metabolites including Pi, PCr and ATP were quantified from the hind limb rat muscle at rest using a high magnetic field strength Bruker 11Tesla/470 MHz spectrometer. A 1.5 x 1.7 cm oval coil, tuned to the frequency of ^{31}P nuclei at the 11T (190.5 MHz) was placed over

the belly of the gastrocnemius muscle. A 3-cm standard ^{1}H surface coil was placed underneath the hind limb to perform shimming and the animal's hind limb was extended such that the calf muscles were centered over the surface coil. Spectra were acquired with a 50 μs square pulse, a TR of 2s, spectral width of 10,000 Hz, 150 averages and 8000 complex data points. In order to account for the differences in the rate of longitudinal relaxation times of phosphate compounds, fully relaxed spectra with a TR of 15 seconds were acquired from non-stimulated rat hind limb muscle. Subsequently, empirical calculations of correction factors (CF) were made by comparing the amplitudes of phosphate spectral peaks at TR of 2s with those at 15s. In concurrence with CF from the rat gastrocnemius muscle in literature (Mizobata, Prechek et al. 1995), the CF for PCr and Pi in our study were 1.40 and 1.69 respectively.

10.4.3.1 ^{31}P-MRS spectral analysis at rest

The spectra were manually phased, and the areas of the β ATP, P_i, and PCr peaks determined by area integration. The enzymatically determined ATP concentration in frozen muscle tissue was taken as the standard to estimate absolute concentration of phosphate metabolites. Absolute Pi and PCr concentrations were determined by using β-ATP as an internal standard and after accounting for correction factors (CF) as follows:

$$[\text{Metabolite}]\ (\mu\text{mol/g wet wt}) = CF_{\text{Metabolite}} \frac{\text{X Integral}_{\text{Metabolite}}}{\text{Integral β-ATP}} \text{X [ATP]}\ (\mu\text{mol/g wet wt}) \quad (10\text{-}1)$$

Intracellular pH was calculated from the chemical shift of the P_i peak relative to PCr using the equation, $pH = 6.75 + \log [(\delta - 3.27) / (5.69 - \delta)]$, where δ is the chemical shift of the P_i peak in ppm. The cystolic phosphorylation potential was calculated in reciprocal form as [Pi][ADP]/[ATP] since the phosphorylation potential itself is not normally distributed. Free

cystolic ADP was calculated from the creatine kinase equilibrium reaction as previously described (Mizobata, Prechek et al. 1995; Thompson, Kemp et al. 1995; Pathare, Vandenborne et al. 2007):

$$[ADP] = \{[\text{free creatine}][ATP]\}/\{[PCr][H+][K_{eq}] \quad (10\text{-}2)$$

Where, the free creatine was quantified by subtracting the PCr content obtained by ^{31}P-MRS from the total creatine content determined biochemically. Intracellular Mg concentration and equilibrium constant (K_{eq}) of the creatine kinase reaction were assumed as 1mM and 1.66 x 10^9 respectively for mammalian skeletal muscle (Veech, Lawson et al. 1979).

10.4.4 Biochemical Assays

Details of ATP analyses are described in chapter 5 (Section 5.2.5.1).

10.4.5 Data Analysis

Repeated measures ANOVA were used to statistically compare the metabolite concentrations, ratios ([Pi]/PCr) and phosphorylation ratios - [ADP][Pi]/[ATP] of the hind limb muscle prior to and over three weeks of spinal cord contusion. All hypotheses were tested at an alpha level of 0.05 and post-hoc Bonferroni corrections were used for multiple comparisons. Analyses were performed using SPSS for Windows, Version 13.3.

10.5 Results

Our data reveal that after one week of spinal contusion, there is marginal elevation in the [Pi]/[PCr] ratios that did not reach statistical significance (p=0.067) (Figure 10-1). A significant elevation in the phosphorylation ratio (1.5fold, p = 0.002) of the paralyzed gastrocnemius muscles was observed at one week after contusion (Figure 10-2). This increase in the ratio after spinal contusion was observed in all, but one animal (Figure 10.3). Specifically, the elevated phosphorylation ratios were accompanied by significant elevations in [ADP] (~52%, p = 0.01) and significant decreases in [PCr] content (13%, p = 0.035) (Table 1, Figure 10-4, Figure 10-5).

Figure 10.3 shows ^{31}P spectra from the gastrocnemius muscle before and after spinal contusion. Note the decrease in the amplitude of PCr peak after spinal contusion in contrast to before injury. No significant change in baseline pH was observed between time points (Table 1). Biochemical analyses revealed no differences in the total creatine and ATP content of gastrocnemius muscle before and after injury. The TCr content (mean±SEM) from gastrocnemius muscle assays was 44.2±2.6 mM and 44.2±3.9 mM for the control and injured group respectively. The ATP content was 4.62±0.44 μmol/g wet weight and 4.63±0.77 μmol/g wet weight from the control and injured gastrocnemius muscles respectively.

Longitudinal follow up of our data show that the phosphorylation ratio at two weeks recovers to 1.38 fold of basal values that recover to control values (1.03times) at three weeks after contusion ($p > 0.01$). The resting [PCr] and [ADP] levels recovered towards normal along the course of three weeks (Table 1).

10.6 Discussion

The main objective of the present study was to quantify the [Pi]/[PCr] ratios in the paralyzed rat gastrocnemius muscle at rest after moderate spinal contusion injury. Results of our study show that there are elevations in the [Pi]/[PCr] ratios, albeit marginal. Additionally, we found drastic elevations in the resting phosphorylation ratios ([ADP][Pi]/[ATP]) of the rat gastrocnemius muscle following one week of moderate spinal contusion. These metabolic alterations recover to baseline values by three weeks after spinal contusion injury.

The [Pi]/[PCr] ratio is a measure of bioenergetic potential of the muscle and has significant bearing to muscle fatigue. In-vitro skinned muscle fiber studies have unanimously reported that elevated inorganic phosphate suppresses skeletal muscle force production (Kentish 1986; Chase and Kushmerick 1988; Martyn and Gordon 1992). Given that the paralyzed skeletal muscle is extremely fatigable and accompanies marked muscle atrophy (Roy, Talmadge et al. 1998; Kjaer,

Mohr et al. 2001; Shields 2002) we expected that the [Pi]/[PCr] ratios in the paralyzed hind limb would increase dramatically. As expected, we found an elevation in the [Pi]/[PCr] ratio (~18%) after one week of moderate spinal cord contusion. This, however, was not statistically significant ($p=0.067$). Nevertheless, based upon the 6.7% chance that the decrease in the ratios is insignificant, we believe that the low statistical power (0.6) of this data might have probably accounted for this insignificance. In fact, following complete spinal cord transection in rats, modest trends towards an increase in the resting Pi/PCr ratios have been reported after one week of injury (Durozard, Gabrielle et al. 2000).

Despite the elevation in Pi/PCr, to our surprise, the increase in the [Pi]/[PCr] ratio observed in our study are largely due to decreases in [PCr] content as versus elevations in [Pi]. Published data from our lab demonstrate a marked elevation in the [Pi]/[PCr] ratio (~75%) of the plantarflexor muscles of mice that are immobilized at the hind limb for two weeks (Pathare, Vandenborne et al. 2007). In animals models of muscle disuse due to denervation induced by crush injuries, Lai et al report an almost 54% increase in the Pi/PCr ratios of the rat gastrocnemius muscle at two weeks after denervation (Lai, Jaweed et al. 1992). In fact, studies have shown that the severity of muscle denervation following the crush injuries dictates the increases in Pi/PCr ratio, with greater increases in the ratios seen after severe injuries (Zochodne, Thompson et al. 1988). Lastly, disuse due to damage to the motor neurons in the spinal cord, as in poliomyelitis, is also associated with an increase in Pi/PCr ratios of the denervated muscle that can get as large as 4fold high (Barany, Siegel et al. 1989). In these studies, elevations are not only significantly higher than that seen in the spinal cord injury models, but the greater elevation in the ratio is accounted predominantly by elevations in [Pi] (Pathare, Vandenborne et al. 2007) or by combinations of elevations in [Pi] and decreases in [PCr] (Barany, Siegel et al. 1989). In

contrast, based on the significant depletion in resting [PCr] content (~13%) as opposed to the less obvious elevations in the [Pi] content (~3% increases) of the paralyzed gastrocnemius muscle, our results support the finding that the [Pi]/[PCr] ratio after spinal contusion is largely influenced by declines in [PCr]. We are unsure why the [Pi] in our study remain unaltered after injury.

Declines in resting [PCr] have also been reported after complete spinal cord transection in the rat gastrocnemius muscle (Durozard, Gabrielle et al. 2000). A decrease in resting PCr content is also observed in patients with muscular dystrophies including severe myotonic, Duchene's and Becker's muscular dystrophies (Taylor, Kemp et al. 1993), mitochondrial myopathy (Taylor, Kemp et al. 1993), hypothyroidism (Taylor, Rajagopalan et al. 1992), hyperthyroidism (Erkintalo, Bendahan et al. 1998) and in chronic fatigue syndrome (Wong, Lopaschuk et al. 1992). Though authors of the above-mentioned studies have not been able to pinpoint the exact cause of depletions in the resting [PCr] in skeletal muscle, the association of the depleted stores in [PCr] to a drastic intolerance to exercise has been well recognized. The authors suggest that the decrease in [PCr] at rest indicates uncoupling of the phosphorylation potential such that the energy available for muscle contraction and other cellular work is decreased (Taylor, Kemp et al. 1993). Similar to muscular dystrophies, SCI is accompanied by marked muscle weakness, atrophy and extreme intolerance to activity (Gerrits, De Haan et al. 1999; Hutchinson, Linderman et al. 2001; van der Salm, Nene et al. 2005).

Irrespective of the mechanism, we observed striking increases in the phosphorylation potential of the paralyzed hind limb muscle after one week of spinal cord contusion. In order to maintain steady state metabolic conditions of a skeletal muscle, increased flux through functional ATPases must be met by an equal increase in ATP synthesis. The driving force, or the link, between cytosolic ATP demand and supply is the phosphorylation ratio [ADP][Pi]/[ATP]. The

free energy for ATP hydrolysis depends on the mass action term of [ADP][Pi]/[ATP]. Accordingly, this ratio is used as a measure of bioenergetic reserve and oxidative metabolism. Small changes in [ATP], [ADP] or [Pi] provide sensitive parameters through which mitochondrial control can be exerted (Klingenberg 1969; Holian, Owen et al. 1977; Veech, Lawson et al. 1979). Mitochondrial respiration is directly regulated by [ADP] or by the phosphorylation ratio - [ADP/[Pi]/[ATP] and the total creatine content only functions to buffer changes in ATP/ADP (Blei, Conley et al. 1993; Kemp, Thompson et al. 1994). An increase in the phosphorylation ratio is more typical of energy demanding states such as exercises. Specifically, during exercises, elevations in [ADP] stimulate ADP entry into the mitochondria to drive mitochondrial respiration (Dudley, Tullson et al. 1987; Houston 2006). The high [ADP][Pi]/[ATP] ratios at rest, as observed in the present study, suggests an uncoupling of the intracellular energy buffer system of the paralyzed hind limb muscle after one week of moderate spinal contusion. As such, the elevated basal [ADP] is reflective of a heightened driving force that most probably triggers respiration at rest after a contusion injury.

The [Pi]/[PCr] phosphorylation ratios are most altered at one week after injury and return to baseline by two weeks of the injury. This reversal of metabolite adaptations of the paralyzed rat gastrocnemius muscle to control values by two weeks after SCI is remarkable - suggestive of the extreme plasticity of the skeletal muscle in response to neural input; but not surprising. Others and we have previously reported an acute response of the skeletal muscle to loss of descending neural input and recovery from this loss in moderate spinal cord injury models (Hutchinson, Linderman et al. 2001; Liu, Bose et al. 2006; Stevens, Liu et al. 2006; Liu, Bose et al. 2008). These studies demonstrate marked atrophy of the paralyzed hind limb muscles as early as one week after spinal contusion; that starts to recover by three weeks. Similarly, Liu et al have

shown acute response in MR relaxation properties as early as one weeks that recovers by three weeks after injury (Liu, Bose et al. 2006). We are not aware of studies that have studied mechanisms related to the reversal of skeletal muscle adaptations after spinal contusion injuries. Nevertheless, given that moderate SCI involves partial loss of descending neural drive, spontaneous motor and physiological recovery after injury is expected. In fact, the return of our animal group to functional levels as evident by normal BBB scores at three weeks after injury, support our speculations.

In conclusion, the cellular capacity for the phosphorylation potential in the paralyzed hind limb muscle, as reflected in an uncoupling of the phosphorylation ratio at rest, is significantly reduced until two weeks of moderate spinal contusion injury in rats. Whether this interferes with the oxidative capacity of the muscle needs further investigation. We explore this inquiry in the next experiment (Chapter 11).

Table 10-1. Absolute phosphate metabolite concentrations before and at various time points (one week, two week, three weeks) after spinal cord contusion in rats. Data are expressed as mean ± standard error.

	Pre SCI	1wk SCI	2wk SCI	3wk SCI
[Pi](mM)	2.46+0.16	2.51+0.18	2.55+0.11	2.21+0.18
[PCr](mM)	26.95+0.84	23.41+1.13*	24.36+0.82	25.81+0.63
Pi/PCr	0.09+0.01	0.11+0.01	0.10+0.004	0.09+0.01
pH	7.14+0.01	7.18+0.01	7.17+0.01	7.17+0.01
[ADP] (μM)	38.10+3.53	57.85+4.81*	51.25+4.08	44.47+2.59
[ADP][Pi]/[ATP] x 10^6M	13.95+1.55	21.31+2.0*	19.26+1.32	14.44+1.14

*Statistically significant differences ($p<0.05$) between pre injury and 1wk post SCI. Abbreviations: [Pi] = inorganic phosphate; [PCr] = phosphocreatine; [ADP] = free cystolic adenosine triphosphate; [ADP][Pi]/[ATP] = phosphorylation ratio.

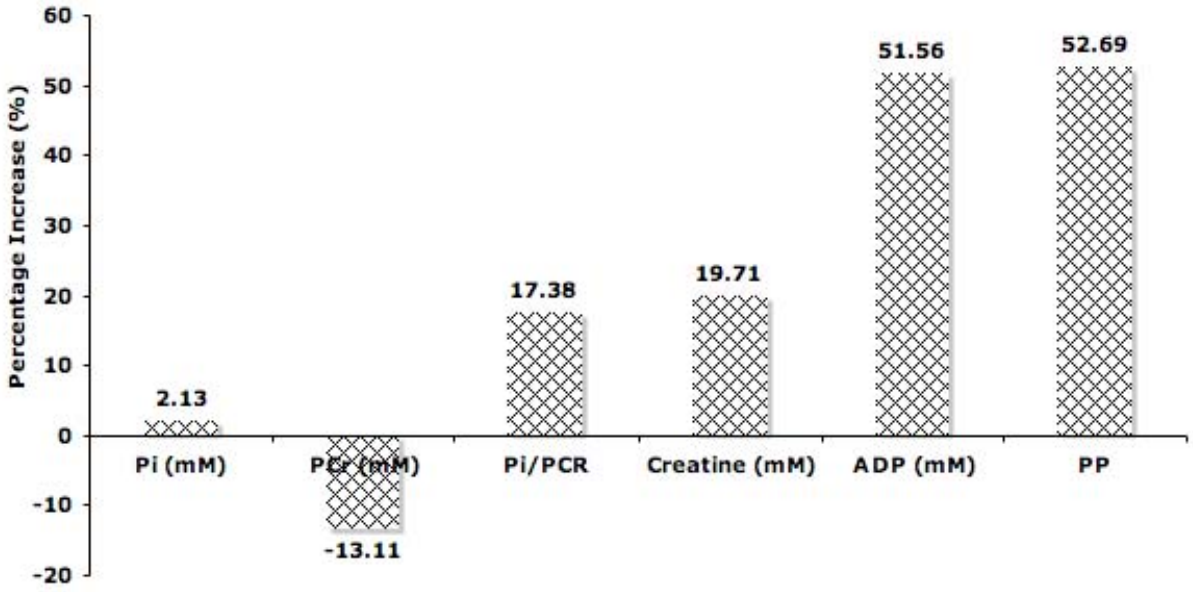

Figure 10-1. Percent change in resting phosphate metabolites of the rat hind limb muscle one week after spinal cord contusion.

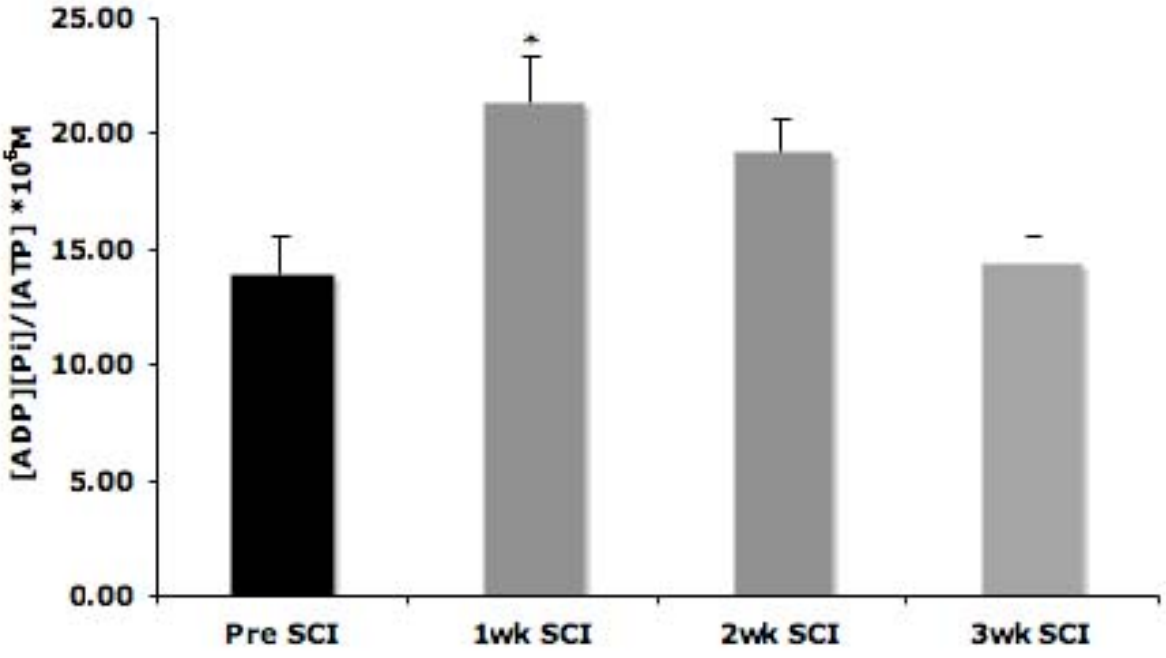

Figure 10-2. Change in phosphorylation ratios - [ADP][Pi]/[ATP] before and after spinal cord contusion.

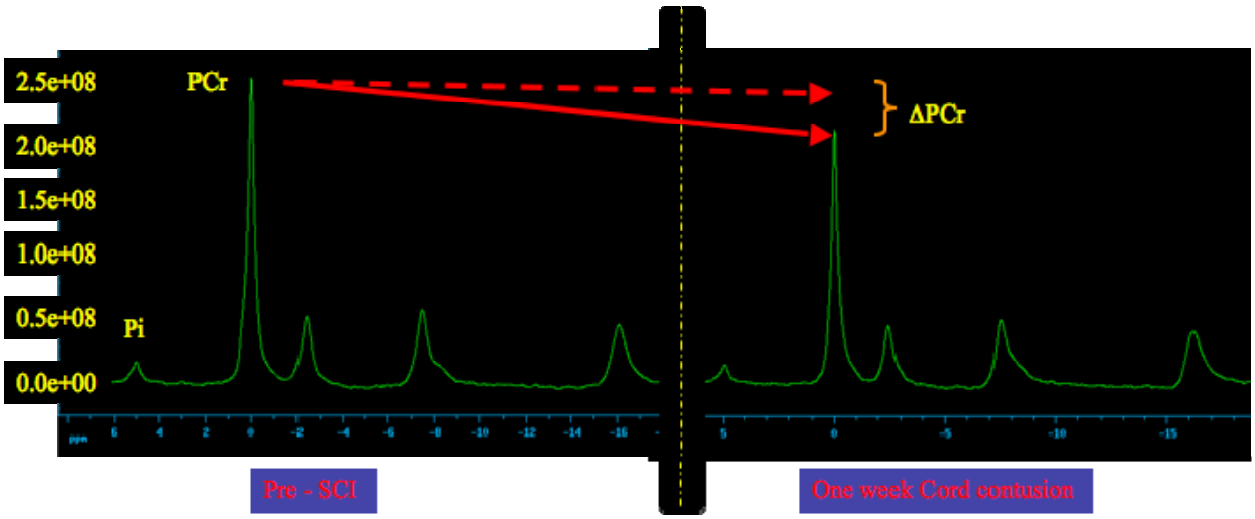

Figure 10-3. Representative ^{31}P-spectra before and after one week SCI obtained at 11T.

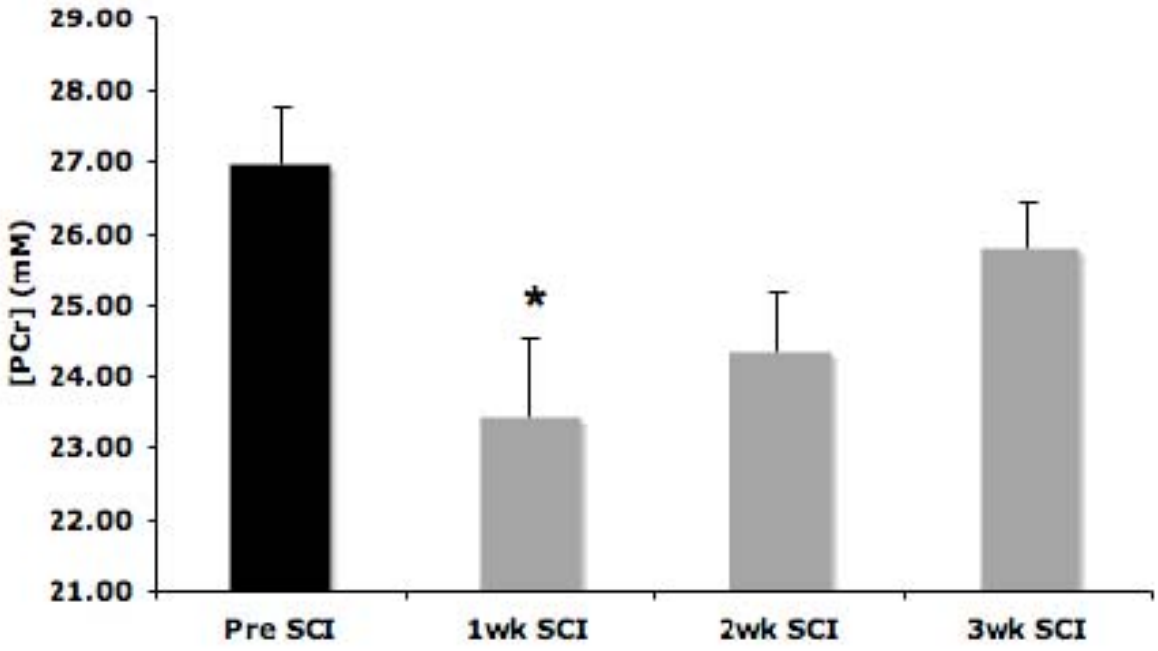

Figure 10-4. Change in [PCr] before and after spinal cord contusion. *Statistically significant difference.

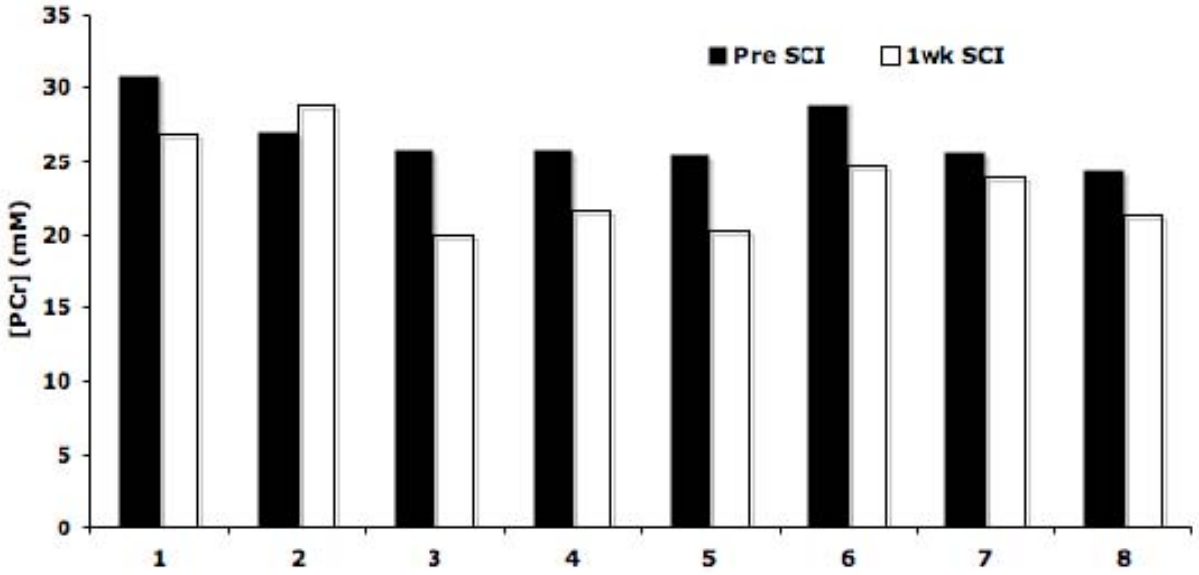

Figure 10-5. [PCr] in individual rat hind limb muscle after one week of spinal cord contusion.

CHAPTER 11
EXPERIMENT SIX - IN-VIVO ASSESSMENT OF SKELETAL MUSCLE BIOENERGETICS AFTER SPINAL CORD CONTUSION IN RATS

11.1 Summary

Declines in skeletal muscle oxidative capacity following spinal cord injury (SCI) have the potential to decrease exercise capacity and negatively impact muscle fatigability. Though altered oxidative capacity after SCI appears to be well documented, most investigators have utilized *in vitro* measurement techniques in their studies. ^{31}P MRS offers a unique non-invasive alternative of measuring oxidative capacity of skeletal muscle and is especially suitable for longitudinal investigations. The purpose of this study was to determine the impact of spinal cord contusion on the oxidative capacity of the rat hindlimb using ^{31}P MRS. Eight young adult female rats were moderately injured at the T8-T10 thoracic spinal cord. ^{31}P MRS measurements were performed at weekly intervals for assessments of oxidative capacity of the rat hindlimb muscle for three weeks. Spectra were acquired in a Bruker 11T/470 MHz spectrometer using a ^{31}P (190.5 MHz) surface coil, placed over the belly of the gastrocnemius muscles. The sciatic nerve was electrically stimulated by subcutaneous needle electrodes with a frequency of 1Hz and a 1ms duration. Kinetic data were collected before, during and after EMS. The PCr area during recovery was fit to a single exponential curve, and the psuedo-first-order rate constant for PCr recovery (k_{PCr}) was determined. As compared to control rats, spinal cord injured rats at one week showed markedly faster PCr depletions rates. Additionally, PCr recovery rates were significantly declined after one week of SCI and regained within two weeks after the injury.

11.2 Introduction

Paralysis or paresis of lower extremity muscles renders persons with SCI to early muscle fatigue and increased energy demands for simple functional activities (Hopman, Dueck et al.

1998; Ulkar, Yavuzer et al. 2003). Skeletal muscle alterations following SCI have the potential to significantly impact daily functional motor performance and locomotor capabilities of persons with SCI that ultimately culminates into long-term disability (Yakura, Waters et al. 1990; Gordon and Mao 1994; Wang, Hiatt et al. 1999). Specifically, declines in skeletal muscle oxidative capacity following the injury have the potential to decrease exercise capacity and negatively impact muscle fatigability (Wang, Hiatt et al. 1999; Bhambhani, Tuchak et al. 2000). Muscle oxidative capacity is defined as the ability of muscle mitochondria to synthesize ATP and is a function of the intrinsic mitochondrial volume, mitochondrial enzyme activation, cystolic redox carriers in mitochondria and vascular oxygen delivery and substrate to the mitochondria (Kemp, Sanderson et al. 1996; McCully, Mancini et al. 1999; Kemp, Roberts et al. 2001). A variety of studies have shown drastic declines in muscle enzyme activity of paralyzed skeletal muscles after complete SCI in humans (Kjaer, Mohr et al. 2001) and in spinalized animal models (Jiang, Roy et al. 1990; Gregory, Vandenborne et al. 2003). In addition, an overall decrease in the mitochondrial DNA content, capillary density and blood flow of the paralyzed muscle, along with declines in skeletal muscle oxygen uptake following exercise are reported following chronic SCI (Scelsi, Marchetti et al. 1982; Barstow, Scremin et al. 1995; Wang, Hiatt et al. 1999; Bhambhani, Tuchak et al. 2000). Unanimously, these alterations are observed in relatively faster muscles such as the gastrocnemius and vastus lateralis, with relatively no metabolic alteration in the slow soleus muscle (Roy, Talmadge et al. 1998; Otis, Roy et al. 2004).

Though altered oxidative capacity after SCI appears to be well documented, most investigators have utilized *in vitro* measurement techniques in their studies. These assessments are not only invasive, but suffer from their inability to yield bio-energetic data from a

functioning muscle in real time. As a result, kinetic changes in muscle metabolism are not best represented using invasive techniques. Moreover, longitudinal follow-up of assessments from the same tissue becomes practically infeasible. Though some investigators have used non-invasive measures such as maximum oxygen consumption (VO_{2max}) as markers of oxidative capacity (McCully, Fielding et al. 1993; Wang, Hiatt et al. 1999), VO_{2max} measures are more global and do not characterize specific muscle metabolic states. Given that skeletal muscle is largely effective in maintaining adequate oxidative ATP formation under most conditions (of rest and exercise), directly studying the cellular events that provide the substrates for ATP synthesis in muscle can prove invaluable.

Magnetic resonance spectroscopy (MRS) seemingly overcomes the limitations that are posed by invasive techniques. In particular, the high spectral resolution (around 1s) enables kinetic assessment of metabolites in real time. In this respect, phosphorus MRS (^{31}P-MRS) has gained tremendous momentum in measuring in-vivo muscle oxidative capacity. One of the most reliable measures of oxidative capacity using ^{31}P-MRS is the rate of PCr recovery after exercise. Studies have established well that the time scale and rates of PCr recovery after exercise intimately match with the time scales and rates of oxygen consumption in the mitochondria following exercise; thereby conferring that PCr resynthesis after exercise is mediated via oxidative phosphorylation (Piiper and Spiller 1970; Meyer 1988; McCully, Iotti et al. 1994; Thompson, Kemp et al. 1995). Accordingly, PCr recovery rates have been conventionally recognized as a non-invasive index of mitochondrial oxidative capacity and extensively used in both healthy and diseased muscles as estimates of muscle oxidative capacity (Levy, Kushnir et al. 1993; Paganini, Foley et al. 1997; McCully, Mancini et al. 1999; Argov and Arnold 2000; Kent-Braun and Ng 2000; Pathare, Vandenborne et al. 2007).

The overall purpose of this study was to non-invasively assess muscle bioenergetics of hind limb muscles after spinal cord contusion in rats. Specifically, we performed longitudinal assessment of the oxidative capacity of rat gastrocnemius muscle for three weeks after spinal contusion using an electrical stimulation protocol and ^{31}P-MRS measures.

11.3 Specific Aims and Hypothesis

11.3.1 Specific Aim

a) To determine the impact of acute spinal contusion (one week) on oxidative capacity of rat hind limb muscle after spinal cord contusion. b) To longitudinally monitor alterations in the skeletal muscle oxidative capacity of the hind limb muscles.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) was performed on the animal hind limb muscle using an electrical stimulation protocol to quantify in-vivo muscle bioenergetics in real time. Measurements were obtained once weekly for three weeks starting at one week post injury.

11.3.2 Hypotheses

a) Immediately after spinal cord contusion (one week), there is a decrease in the oxidative capacity of the paralyzed hind limb muscle.

b) Muscle oxidative capacity approach recovery by three weeks of spinal cord contusion.

11.4 Methods

11.4.1 Experimental Design

Two groups of randomly distributed adult rats were studied. Animals either underwent a spinal cord contusion (n=8) or served as controls for wet lab procedures relevant to the study (see below). The spinal injured group underwent ^{31}P-MRS measurements prior to injury and at weekly intervals for three weeks after injury.

11.4.2 Animals

Sixteen adult Sprague Dawley female rats (12 week, 228-260g; Charles River, NJ) were housed in a temperature controlled room at 21^0C with a 12:12 hours light: dark cycle and provided with rodent chow and water *ad libitum*. Of these, 8 rats were moderately injured at the T8-T10 thoracic spinal cord levels and 8 rats served as controls in providing healthy muscle tissue to run biochemical assays for the quantification of phosphate compounds. The control group also provided reproducibility results of our ^{31}P data. All experimental procedures were performed in accordance with the U.S. Government Principle for the Utilization and Care of Vertebrate Animals by approval of the Institutional Animal Care & Use Committee at the University of Florida.

Details about spinal cord contusion operation procedures have been described in detail in chapter 5 (Section 5.2.2)

11.4.3 Data Collection

Combinations of an electrical stimulation and ^{31}P-MRS were used to determine the in-vivo skeletal muscle oxidative capacity of the rat gastrocnemius muscle before and after spinal cord contusion injuries.

11.4.3.1 Experimental electrical stimulation protocol

An electrical muscle stimulation protocol was adopted to determine the mitochondrial oxidative capacity of the rat hind limb muscle *in-vivo*. Animals were anesthesized using gaseous isoflurane in oxygen (3% box induction), and maintained at 0.5%-2.5% during the MR procedures. The limb was shaved and cleaned with alcohol and a circular ^{31}P (190.5 MHz) tuned surface coil was placed over the belly of the gastrocnemius muscle. A ^{1}H surface coil was placed underneath the hind limb to perform shimming. Two needle electrodes were placed subcutaneously – one over the region of the third lumbar vertebrae and the other land marked

over the greater trochanter - to stimulate the hind limb plantarflexor muscles via stimulation of the sciatic nerve. Electrical stimulation was carried out for four to six minutes to deplete PCr by ~30 to ~40%. A Grass Stimulator (Quincy MA) with a Grass Model SIU8T stimulation isolation unit (Grass Instruments, West Warwick, RI) was used to deliver a monophasic, rectangular pulse with a 1ms pulse duration, 1Hz frequency and 10V. Following the electrical stimulation, the muscle was allowed to recover for twenty minutes. During the entire duration of stimulation and recovery, spectra were collected. No attempts were made to synchronize the radiofrequency pulse with the muscle stimulation. The FIDs were multiplied by an exponential corresponding to a 25Hz line broadening. Vital signs of the animal were monitored throughout the experimental procedure.

11.4.3.2 ^{31}P magnetic resonance spectroscopy

The above electrical stimulation protocol along with combination of ^{31}P-MRS was used to assess the oxidative capacity of the hind limb rat muscle in a Bruker 11Tesla/470 MHz spectrometer. A 1.5 x 1.7 cm oval surface coil tuned to ^{31}P (190.5 MHz) was placed over the belly of the gastrocnemius muscle. A 3-cm standard ^{1}H surface coil was placed underneath the hind limb to perform shimming and the animal's hind limb was extended such that the calf muscles were centered over the surface coil. Spectra were acquired with a 50 μs square pulse, a TR of 2s, spectral width of 10,000 Hz, 10 averages and 8000 complex data points. Spectra were averaged into 20s bins and acquired at rest (5 min), electrical stimulation (4-6 min), and recovery (20 min); thus amounting to a total of 93 fids. The partially relaxed spectra were then calibrated by comparison with fully relaxed spectra (acquired at TR of 15seconds) to determine correction factors (CF).

11.4.4 ^{31}P magnetic resonance spectroscopy: data analysis

The electrical stimulation protocol depletes PCr from skeletal muscle until stimulation is on and MRS obtains this kinetic information in real time. Dynamic changes in PCr levels were measured using complex principal component analysis (Elliott, Walter et al. 1999). Recovery data were fitted to a single exponential curve, and the pseudo first-order rate constant for PCr recovery (k_{PCr}) was determined (Meyer 1988). End exercise [Pi] and [PCr] were measured by area integration using 2D Bruker WinNMR data processing software. The maximal rate of PCr resynthesis, a measure of mitochondrial oxidative capacity ($V_{max\text{-}lin}$) was calculated based on k_{PCr} and baseline PCr values ($V_{max\text{-}lin} = k_{PCr} . [PCr]_{rest}$) (Walter, Vandenborne et al. 1997). Initial rates of PCr recovery (V_{meas} mM/min, a direct measure of mitochondrial ATP synthesis) was determined from the first three to four data points in recovery; depending upon the best linear curve fit. The initial rate of PCr resynthesis was also extrapolated from the product of k_{PCr} and amount of PCr depletion (ΔPCr). Thus, V_{ex} (mM/min) = k_{PCr}. ΔPCr (Walter, Vandenborne et al. 1997).

Rates of PCr depletion at onset of stimulation (V_{dep} mM/min, a measure of ATP demand) were determined from the first three to nine data points (first through three minutes) of PCr declines during stimulation.

The maximum oxidative ATP synthesis rate (Q_{max}), which is a function of intrinsic mitochondrial content and enzyme activity, oxygen and substrate supply to the mitochondrion, and cytosolic redox state (Kemp, Sanderson et al. 1996), was calculated from the known hyperbolic relationship between k_{PCr} and cytosolic free [ADP] and from k_{PCr} and [ADP][Pi]/[ATP].

$Q_{max\text{-}ADP} =$ Vmeas (1 + Km/[ADP]) mM/min, where Km is the Michaelis constant and is assumed as 50 μM for rat leg muscle (Thompson, Kemp et al. 1995).

$Q_{max\text{-}[ADP][Pi]/[ATP]}$ = Vmeas (1 + Km/[ADP][Pi]/[ATP]) mM/min, where Km is assumed as 0.11 mM.

11.4.5 Biochemical Assays

Details of biochemical analyses are described in chapter 5 (Section 5.2.5.1).

11.4.6 Statistical Analysis

Repeated measures ANOVA were used to statistically compare outcome measures from kinetic data at rest, during electrical stimulation and during recovery. We compared the three-minute V_{dep}, end exercise metabolite concentrations, V_{meas}, V_{ex}, k_{PCr} and maximum mitochondrial capacities (V_{max} measures) and oxidative ATP synthesis rates (Q_{max} measures) of the rat hind limb muscle prior to and along three weeks of spinal cord contusion. All hypotheses were tested at an alpha level of 0.05. Post-hoc Bonferroni corrections were used for multiple outcome comparisons. Analyses were performed using SPSS for Windows, Version 13.0.1.

11.5 Results

Dynamic relative changes in phosphate peaks in response to our stimulation protocol are represented in Figure 11-1. Overall, results of our data show drastic differences between the pre and post-injury outcome measures at one week after SCI (Table 1). Figure 11-2 shows the average depletion and recovery graphs before and after one week of injury. For purposes of data presentation, average results of our kinetic data are described separately in three steps as - data at onset of stimulation, at end exercise and during recovery.

Kinetic changes at onset of EMS: The absolute rates of PCr depletion (V_{dep}) at three minutes of electrical stimulation were significant faster ($p=0.02$, by ~30%) after one week of spinal contusion than before injury (Figure 11-3, 11-4). V_{dep} at earlier times (one and two

minutes) did not reach statistical significance (Figure 11-3). While before injury, the time required to deplete PCr was six minutes, after injury the PCr depleted to similar or more amounts in around 4 minutes (Figure 11-4).

Kinetic changes at the end of EMS: The percentage PCr drop in the injured group was lower by 39% in the injured group as versus the percentage drop before injury (0.41±2 % of resting values versus 30±3% of resting values, p=0.022). Delta [PCr] values were however not different between groups (Table 11-1). Due to large differences in baseline [PCr] after spinal contusion injury at one week, the end stimulation [PCr] was lower by 30% (p=0.012) and end stimulation [ADP] greater by 67% (p=0.021) after one week of injury (Table 11-1). Additionally, this significantly affected end exercise [ADP][Pi]/[ATP] ratios (p = 0.011). The end pH after stimulation was lower in the one week injured group by 0.05 units as compared to before injury (6.98 versus 7.02 pH units). Importantly, end pH did not decrease by more than 0.2 units as compared to before stimulation throughout the experiment.. End stimulation [Pi] + [PCr] taken together were not different from baseline values of [Pi] + [PCr] taken together.

Kinetic changes during recovery: Following one week of spinal contusion, there was a significant reduction (~24%) in k_{PCr} measures of the paralyzed gastrocnemius muscle (p=0.001) (Figure 11-5). However, no significant alterations were seen in V_{meas} and V_{ex} measures. Consequently, maximum mitochondrial capacities (V_{max}, V_{meas} and V_{ex}) and maximum mitochondrial ATP synthesis rates (Q_{max}) were either significantly different or unaltered after the injury (Figure 11-6, Figure 11-9, and Table 11-1). Moderate correlation (r=0.5) was seen between V_{max} and V_{dep} (Figure 11-7) and no correlation observed between end [ADP] and V_{meas} (r=0.016) (Figure 11-8).

All the measures that changed at one week, returned to near baseline values by three weeks after injury. Biochemical assay results showed that there were no significant differences in the [ATP] and [TCr] in the gastrocnemius muscle before and after spinal contusion injury. Biochemical analyses revealed no differences in the total creatine and ATP content of gastrocnemius muscle before and after injury. The TCr content (mean±SEM) values from gastrocnemius muscle assays were 44.2±2.6 mM and 44.2±3.9 mM for the control and injured group respectively. The ATP content was 4.62±0.44 μmol/g wet weight (~ 6.72mM) and 4.63±0.77 μmol/g wet weight (~6.85mM) from the control and injured gastrocnemius muscles respectively.

11.6 Discussion

In the present work, we utilized combinations of an electrical stimulation protocol and ^{31}P-MRS to determine the *in-vivo* skeletal muscle bioenergetics of the rat hind limb muscle during exercise after moderate thoracic spinal cord contusion injury. The key results of our study show that there is a considerable elevation (~30%) in the PCr depletion rate (V_{dep}) and a decrease (~24%) in the PCr resynthesis rate constants (k_{PCr}) of the paralyzed muscle at one week after injury that return to near normal values by three weeks. Our data suggests that there is a significant imbalance in the energy producing and/or consuming states of the paralyzed muscle along with a decrease in the overall mitochondrial oxidative capacity for oxidative phosphorylation after one week of contusion injury.

An important and novel finding of our present study is that at one week after spinal cord contusion, PCr depletes faster and to relatively lower levels than the pre-injury levels. Specifically, with the protocol used in the study, the V_{dep} at one minute after electrical stimulation is similar before and at after one week after the injury. However, by two minutes of electrical stimulation, the PCr levels of the contused group begin to drop more rapidly and lower

as compared to that seen before the contusion. With the electrical stimulation parameters used in the present study, duration of 6minutes was set to deplete the PCr content in our control group (pre-injury) by ~32%. However, after spinal contusion, we had to stop the stimulation at 4minutes, because by this time the PCr had depleted by ~42% and any further PCr depletion was necessary to avoid. Moreover, while the control group reached a clear steady state by almost three minutes into stimulation, the injured group never reached steady states. Parallel to our findings, similar rapid and farther V_{dep} rates for a given exercise protocol have been documented in a variety of disease states including chronic fatigue syndrome (Wong, Lopaschuk et al. 1992) myotonic dystrophy (Taylor, Kemp et al. 1993), peripheral vascular diseases (Kemp, Hands et al. 1995), denervation (Hayashi, Ikata et al. 1997) and chronic heart failure (Toussaint, Kwong et al. 1996). We suppose that the likely mechanisms for the metabolic response of the paralyzed muscle might be secondary to increases in ATP demand to perform similar work, a decrease in local blood perfusion that impacts ATP synthesis via oxidative phosphorylation or a combination of both. Our viewpoint is supported by the following explanations.

Homeostatic mechanisms within the myocyte couple overall ATP utilization with ATP synthesis; thereby maintaining nearly steady concentrations of ATP during low intensity exercises (Erecinska and Wilson 1982; Kushmerick 1995). Specifically, during muscle contraction, increasing ATP demands in the myocyte are met by PCr breakdown via the creatine kinase equilibrium reaction. This decrease in PCr content indicates the energy buffer role of PCr, and consequently of the phosphorylation ratio ([ADP][Pi]/[ATP]) in response to ATP demands. Accordingly, the magnitude of change in PCr concentration reflects the demand for oxygen and substrates, and PCr depletion rates are purported to measure the ATP needed to meet cellular demands (Kemp, Hands et al. 1995; Toussaint, Kwong et al. 1996). The finding that more

amount of PCr is utilized for hydrolysis to ATP implies that the ATP demands for similar intensities of muscle contraction are higher than pre-injury levels. Indeed, sufficient evidence in literature reveals that the paralyzed skeletal muscle is predisposed to increased fatigability, deconditioning, declines in isometric force production over repetitive bouts of contraction and decreases in muscle endurance (Gerrits, De Haan et al. 1999; Shields 2002). Consequently, the paralyzed muscle will function less economically than normal and require more energy to perform a given contraction. Moreover, increases in ATP consumption of paralyzed muscle can also result from ATP consuming events in the tissue such as muscle atrophy (Erkintalo, Bendahan et al. 1998) and the uncoupling of oxidative phosphorylation (Taylor, Kemp et al. 1993; Scheuermann-Freestone, Madsen et al. 2003) – both of which are muscle adaptations after spinal cord contusion that have been presented separately from past data in our lab (Liu, Bose et al. 2008) and in the current study respectively. Taken together, our findings of an apparent increase in ATP closely matches with the functional impairments posed by paralyzed muscles and most likely provides a mechanistic explanation to those findings. However, confirmation from actual measurements of the energy cost of contractions by the amount of ATP produced for a given power output (Russ, Elliott et al. 2002) in the rat model of moderate spinal contusion are warranted.

Another explanation for the faster V_{dep} after SCI is an apparent decrease in the local blood perfusion that interferes with ATP synthesis via oxidative phosphorylation during muscle contraction. During steady state exercises, there are increases in oxidative phosphorylation rates; which are supplemented by an increase in the local blood perfusion. Marro et al have shown in the rat hind limb muscle that during a range of low-level contractile activity, the declines in PCr concentrations are the mirror image of the perfusion increases (Marro, Olive et al. 2007). A

correlation exists between energy metabolism and oxygen supply that is fueled by local blood circulation in rat skeletal muscles during exercise (Idstrom, Subramanian et al. 1984; references from Olive, Dudley et al. 2003). Steady state exercises are met by an initial rapid increase in blood flow till oxygen delivery matches the exercise demands and then plateaus (Kushmerick 1981; Van Beekvelt, Shoemaker et al. 2001). Studies have shown that these initial rapid increases in blood flow, in turn, depend upon muscle contractile efficiency and vasomotor tone of the capillary pool (Tschakovsky, Shoemaker et al. 1996; Van Beekvelt, Shoemaker et al. 2001). Noticeably, these data imply that an inadequate blood flow during exercise will result in inadequate delivery of oxygen to the contracting muscle cells and limit the ability of mitochondria to produce ATP via oxidative phosphorylation (denervation paper). Indeed, after SCI in humans, Olive et al have shown that there is an approximate five-fold increase in the time to peak blood flow and a significant delay in the blood flow response at the onset of muscle stimulation in persons with chronic SCI (Olive, Dudley et al. 2003). This increased time of blood delivery, in turn, significantly compromises the ability of the paralyzed muscle to meet the energy demands of muscle contraction secondary to electrical stimulation. Various mechanisms for this deficiency in blood flow have been addressed including alterations in the vasomotor tone, decreased contractile ability of the paralyzed muscle to deliver blood, decreased oxidative capacity secondary to shift in muscle fiber type composition towards fast glycolytic fibers and a decreased hyperemic response to muscle contractions (Olive, Dudley et al. 2003). Similar to humans, the paralyzed mammalian rat muscle exhibits significant levels of muscle atrophy (Hutchinson, Linderman et al. 2001; Liu, Bose et al. 2008), increase in the number of type II glycolytical fibers of the paralyzed hind limb muscles (Stevens, Liu et al. 2006), alterations in *in-vitro* measures of oxidative capacity (Gregory, Vandenborne et al. 2003), altered contractile

properties (Stevens, Liu et al. 2006) and vasomotor insufficiency (Guizar-Sahagun, Velasco-Hernandez et al. 2004; Laird, Finch et al. 2008). Lastly, other disease states (including denervation, heart failure and peripheral nerve diseases) that have shown sizably compromised V_{dep} rates using similar in-vivo measures as ours, have demonstrated that the local perfusion to the involved muscle is compromised; thereby suggesting that the oxygen delivery to the muscle is a likely contributor of the abnormal metabolic response seen (Kemp, Hands et al. 1995; Toussaint, Kwong et al. 1996; Hayashi, Ikata et al. 1997). In view of these findings, we deduce that inherent characteristics of the paralyzed muscle potentially interfere with the local blood perfusion, which in turn hampers the oxygen supply to the exercising muscle. Consequently, ATP production from oxidative phosphorylation during exercise is compromised and is reflected as faster V_{dep} rates.

Another principal finding of our study is that the rate constants of PCr recovery (k_{PCr}) are significantly decreased after one week of spinal contusion. Studies have well established that during recovery from exercise, glycolysis ceases and PCr in the muscle cell is replenished at the expense of ATP produced via mitochondrial oxidative phosphorylation (Taylor, Bore et al. 1983; Meyer 1988; Quistorff, Johansen et al. 1993; Kemp, Roberts et al. 2001; Mattei, Bendahan et al. 2004). Accordingly, k_{PCr} has been extensively used in both healthy and diseased muscles as estimates of muscle oxidative capacity (Meyer 1988; Paganini, Foley et al. 1997; McCully, Mancini et al. 1999; Argov and Arnold 2000; Kent-Braun and Ng 2000; Pathare, Vandenborne et al. 2007). The declines in k_{PCr} of the paralyzed muscle in the present study are parallel to various disease states including the rat model of spinal transection (Durozard, Gabrielle et al. 2000), complete SCI in humans (Levy, Kushnir et al. 1993; Hartkopp, Harridge et al. 2003), peripheral vascular diseases (Kemp, Hands et al. 1995) and models of muscle disuse including

immobilization in mice (Pathare, Vandenborne et al. 2007) and hind limb suspension in rats (Yoshida, Ikata et al. 2001). In comparison with the percentage decline in the PCr recovery rates of the paralyzed hind limb muscle after spinal contusion (-33 % decline in T1/2 recovery rates at one week), an almost 134% decline in T1/2 is reported after 60 days of spinal cord transection. We believe that these discrepancies are largely due to the difference in severities of the two types of cord injuries. Moreover, unlike our study, the pH in the transection study was not controlled. As a result, intracellular acidosis at the end of stimulation might have slowed down the rates of PCr recovery after spinal transection. When measuring oxidative capacity using MRS, it is essential that the muscle does not get acidotic. This is because pH declines below 6.75 units (Taylor, Bore et al. 1983; McCully, Iotti et al. 1994; Boesch 2007) significantly impacts k_{PCr} rates and cannot adequately reflect skeletal muscle oxidative capacity. In our present study, the end stimulation pH was 6.9 and did not decrease by more than 0.2 units as compared to the baseline pH. We could achieve this by trading against shorter periods of electrical stimulation in our injured group. Importantly, our control data show similar k_{PCr} as those reported in literature from healthy rat gastrocnemius muscles (Meyer 1988; Paganini, Foley et al. 1997). Collectively, the decline in k_{PCr} r and hence the V_{max} measures in our present study suggest an overall decrease in mitochondrial oxidative capacity of the paralyzed rat gastrocnemius muscle.

The maximum oxidative ATP synthesis rate (Q_{max}), is a function of intrinsic mitochondrial content and enzyme activity, oxygen and substrate supply to the mitochondrion, and cytosolic redox state (Kemp, Sanderson et al. 1996). Cytoplasmic [ADP] plays a crucial role in the respiratory control in skeletal muscle; such that the Q_{max} largely depends on [ADP] in a Michaelis-Menten fashion (Chance, Leigh et al. 1985; Kemp and Radda 1994; Paganini, Foley et al. 1997). According to this model, Q_{max} derived from the known hyperbolic relationship

between k_{PCr} and cytosolic free [ADP] adequately represents maximum oxidative ATP synthesis rate (Paganini, Foley et al. 1997). Following one week of spinal cord contusion, various indices of the PCr resynthesis rates including V_{max}, $Q_{max\text{-}[ADP]}$ and $Q_{max\text{-}[ADP][Pi]/[ATP]}$ are reduced; thereby suggesting that mitochondrial ATP production is considerably impaired in a paralyzed muscle. In spite of an elevation in the end stimulation mitochondrial driving force ([ADP][Pi]/[ATP]), we encountered a significant decline in the Q_{max} measures in our one week injured group. This most likely suggests that the driving force is unable to trigger an adequate mitochondrial response in enhancing oxidative phosphorylation. We believe that this is most likely due to a possible uncoupling between oxygen fluxes in the mitochondria and subsequent ATP synthesis rates. In fact, the finding from our present study that lower maximum mitochondrial capacity rates (V_{max}) accompany faster rates of PCr depletion rates (good negative correlation between V_{max} and V_{dep}, r=0.5, Figure 11-7) supports our view.

Our finding that the mitochondrial oxidative capacity of the partially paralyzed skeletal muscle is compromised, is in concurrence with in-vivo and in-vitro models of complete SCI in both humans and animal models. In an animal model of spinal transection, Durozard et al have used non-invasive magnetic resonance spectroscopy to demonstrate declines in oxidative capacity of the gastrocnemius muscle in rats. Based upon drastic decreases in both k_{PCr} and Q_{max} measures, the authors suggest transition in the source of energy supply from oxidative to anaerobic pathways for muscle metabolism following complete paralysis of the rat hind limb muscle (Durozard, Gabrielle et al. 2000). Additionally, in vitro studies show that mixed muscle such as the gastrocnemius and the vastus lateralis has significantly lower mitochondrial enzyme activity following spinalization in both cats and rats (Jiang, Roy et al. 1990; Gregory, Vandenborne et al. 2003). In addition, an overall decrease in the mitochondrial DNA content,

capillary density and blood flow of the paralyzed muscle, along with declines in skeletal muscle oxygen uptake following exercise are reported following chronic SCI (Scelsi, Marchetti et al. 1982; Barstow, Scremin et al. 1995; Wang, Hiatt et al. 1999; Bhambhani, Tuchak et al. 2000). Various mechanisms including skeletal muscle atrophy, change in muscle fiber type composition, muscle inactivation and muscle deconditioning (as reflected in decreased functional activity and limitation of exercise tolerance to daily activities) have been recognized as precipitating factors in compromising the oxidative capacity of the paralyzed skeletal muscle. In fact, since mitochondrial function can be impacted under low perfusion conditions such as those experienced in peripheral vascular disease (Isbell, Berr et al. 2006; Greiner, Esterhammer et al. 2007; Kramer 2007) and heart failure (Toussaint, Kwong et al. 1996), deficits in local blood perfusion after SCI (Olive, Dudley et al. 2003; Guizar-Sahagun, Velasco-Hernandez et al. 2004) as a potential contributor to mitochondrial oxidative capacity cannot be ruled out.

Similar to rate constants of PCr recovery, initial rate of PCr recovery (V_{meas}) has been demonstrated to reflect mitochondrial oxidative capacity (Meyer 1988; Kemp, Thompson et al. 1994; Lodi, Kemp et al. 1997) and more specifically is an estimate of the end-exercise rate of oxidative ATP synthesis (Kemp G, Hands L et al 1995). To our surprise however, we do did not encounter simultaneous declines in V_{meas} from our data at one-week post injury. We believe that several factors could account for this discrepancy - a) A decreased sensitivity of V_{meas} as versus k_{PCr} : k_{PCr} is calculated after fitting several points in recovery (60 fids in our data set) to a single exponential function; whereas V_{meas} is estimated from the first two-three data points in recovery. Accordingly, spectral noise from V_{meas} calculations leads to larger variability in V_{meas} calculations (CV=24%) than the k_{PCr} calculations (CV=21%); thereby rendering V_{meas} as a relatively less sensitive measure. b) Biexponential function of k_{PCr} recovery rates: V_{meas} values

are based on a linear model of recovery while k_{PCr} is extrapolated from a monoexponential function. Given that both measures reflect similar physiological mechanisms in the skeletal muscle, it is tempting to question if the recovery curves follows a biexponential function. However, from careful visual inspection of our PCr recovery graphs (that show excellent monoexponential fits; R^2= 0.97 – 0.99) we do not believe that to be the case. c) End stimulation elevations in [ADP]: At the end of electrical stimulation, the [ADP] is significantly elevated in injured animals as versus before injury. Studies have shown that elevated [ADP] levels serve as a strong respiratory drive (Kushmerick 1981; Paganini, Foley et al. 1997) and exert an influence in enhancing the rates of PCr recovery via increased ATP synthesis. In fact, for similar rates of V_{meas}, we can infer from the Michelis-Mentel relationship between V_{meas} and [ADP] that the maximum rate of oxidative ATP synthesis (Q_{max}) should be higher in our injured group than before SCI. However, our data show tendencies towards a decline in the Q_{max} measures when predicted from both the V_{meas} and V_{ex} measures. Additionally, our data do not show any correlation between end [ADP] and V_{meas} (Figure 11-8). Collectively, we believe that the V_{meas} rates are most likely the result of an increased variability in our measurements.

The V_{dep} and V_{max} measures in our study are predominantly altered at one week after injury and return to baseline by two weeks of the injury. This reversal of metabolite adaptations of the paralyzed rat gastrocnemius muscle to control values by two weeks after moderate spinal contusion injury is remarkable - suggestive of the extreme plasticity of the skeletal muscle in response to neural input - but not surprising. Others and we have previously reported an acute response of the skeletal muscle to loss of descending neural input and recovery from this loss in moderate spinal cord injury models (Hutchinson, Linderman et al. 2001; Liu, Bose et al. 2006; Stevens, Liu et al. 2006; Liu, Bose et al. 2008). These studies demonstrate marked atrophy of the

paralyzed hind limb muscles as early as one week after spinal contusion; that starts to recover by three weeks. Similarly, Liu et al have shown acute response in MR relaxation properties as early as one weeks that recovers by three weeks after injury (Liu, Bose et al. 2006). We are not aware of studies that have studied mechanisms related to the reversal of skeletal muscle adaptations after spinal contusion injuries. Nevertheless, given that moderate SCI involves partial loss of descending neural drive, spontaneous motor and physiological recovery after injury is expected.

We recognize potential limitations of the current study. We did not obtain force mechanics data in these experiments to determine the intensity of stimulation voltage used for our muscle stimulation protocol. However, the voltage was adjusted to give maximum contraction of the gastrocnemius muscle as confirmed by palpation of the muscle and the stimulation parameters kept constant throughout the experiment. Moreover, the protocol we used in the study is well documented in literature and is established to stimulate the gastrocnemius, soleus and plantaris muscle groups (Meyer, Brown et al. 1985; Meyer 1988; Paganini, Foley et al. 1997). In fact, results from our pilot study show that the coil penetration was enough to obtain signal, predominantly from the gastrocnemius muscle. Another reservation of the study is that fiber atrophy might have caused the signal to bleed from surrounding slow muscle tissue (such as the soleus); in which case the k_{PCr} tend to slow down. However, the soleus muscle is a very small part of the rat gastrocnemius-plantaris-soleus muscle complex and we expect minimal contribution, if any, of the soleus muscle to our spectral signal. Moreover, previous reports from our lab have demonstrated that muscle atrophy continues for at least two weeks after moderate spinal contusion (Stevens, Liu et al. 2006; Liu, Bose et al. 2008). Since k_{PCr} in our present study recover to normal at two weeks after injury in spite of the atrophy, the lower recovery rates due to atrophy alone are most likely ruled out. Lastly, in extrapolating the in-vivo absolute

concentrations of phosphate metabolites, the in-vitro [ATP] and [TCr] measures obtained at three weeks after injury were assumed to be constant at all time points after injury. However, based upon studies in complete spinal cord transection in rats, Durozard et al do not show any alterations in the [ATP] in the paralyzed gastrocnemius muscle at day 7, 30 and 60 after the injury (Durozard, Gabrielle et al. 2000). Whether muscle [TCr] changes along the course of spinal cord contusion injury needs to be verified in future studies.

In conclusion, results of our study show striking declines in the maximum mitochondrial capacity and alteration in the ATP supply and/or demand characteristics of the paralyzed gastrocnemius after one week of spinal cord contusion. By using *in-vivo* magnetic resonance spectroscopic measures in the study, we have been able to circumvent limitations posed by invasive and global measures of assessing the oxidative capacity of skeletal muscle. Specifically, the non-invasiveness of ^{31}P-MRS enabled us to longitudinally assess the cellular events that define muscle oxidative capacity in real time and with high spectral resolution. Overall, our data suggests an oxidative metabolic defect in the paralyzed hind limb muscle that might potentially contribute to the host of motor dysfunctions seen after SCI (Waters, Adkins et al. 1994; Ulkar, Yavuzer et al. 2003). Our findings support the potential usefulness of therapeutic interventions aimed at improving aerobic muscle metabolism after this injury.

Table 11-1. Kinetic ^{31}P-MRS data from the rat gastrocnemius muscle. Average data (mean±standard error) during exercise and recovery from exercise is presented before and for three weeks after moderate spinal contusion injury (n=8).

	Pre SCI	1wk SCI	2wk SCI	3wk SCI
I) Data at onset of EMS				
3min [PCr] V_{dep} ($mM.min^{-1}$)	-2.44±0.18	-3.16±0.18*	-2.42±0.22	-2.68±0.22
Baseline [PCr] + [Pi] (mM)	29.40+0.86	25.92+1.23*	26.91+0.89	28.01+0.67
II) End EMS data				
End ex pH	7.02±0.01	6.97±0.02*	6.99±0.02	7.00±0.01
End ex [PCr] (mM)	21.79±1.25	15.24±0.96*	19.55±1.24	19.03±0.50
End ex [Pi] (mM)	9.88±0.81	9.59±0.76	9.03±0.87	9.52±0.68
End ex [ADP] (μM)	47.11±4.46	78.67±8.02*	53.89±5.31	54.43±2.55
End ex [ADP][Pi]/[ATP]	67.77±7.18	110.12±12.51*	74.38±12.97	77.06±6.25
Delta [PCr] (mM)	6.48±0.88	8.17±0.69	5.74±0.99	7.09±0.71
Percentage PCr drop	30±2.7%	41±2%*	35±4%	34±4%
III) Recovery data				
a) PCr recovery constants				
k_{PCr} (min^{-1})	0.65±0.05	0.50±0.06*	0.67±0.05	0.63±0.07
1/k (s)	96±8	134±17*	94±8	108±18
Initial recovery rate (min^{-1})	0.24±0.02	0.26±0.01	0.28±0.06	0.27±0.01
b) Mitochondrial Oxidative Capacity (V)				
$V_{max\text{-}lin}$ ($mM.min^{-1}$)	17.52±1.52	11.64±1.42*	16.25±1.28	15.97±1.59
V_{meas} ($mM.min^{-1}$)	6.49±0.65	6.13±0.40	5.90±1.71	6.96±0.31
V_{ex} (mM.min-1)	4.38±0.75	3.96±0.50	3.60±0.51	4.33±0.66
c) Oxidative ATP synthesis rates (Q_{max})				
Based on k_{PCr}				
$Q_{max\text{-}ADP}$ ($mM.min^{-1}$)	27.34±1.98	15.94±2.24*	24.42±2.15	23.14±2.34
$Q_{max\text{-}ADP*Pi/ATP}$ ($mM.min^{-1}$)	47.88±4.67	24.93±3.79*	46.21±6.11	39.41±4.69
Based on V_{meas}				
$Q_{max\text{-}ADP}$ ($mM.min^{-1}$)	10.27±1.03	8.39±0.85	8.46±2.31	10.22±0.45
$Q_{max\text{-}ADP*Pi/ATP}$ ($mM.min^{-1}$)	17.56+1.72	12.71+1.34*	16.09+2.99	17.28+0.84
Based on V_{ex}				
$Q_{max\text{-}ADP}$ ($mM.min^{-1}$) (V_{meas})	5.70±1.30	5.39±0.75	4.57±0.92	6.28±0.91
$Q_{max\text{-}ADP*Pi/ATP}$ ($mM.min^{-1}$)	9.89+2.32	8.41+1.30	8.59+0.98	10.48+1.46

*Statistically significant differences ($p<0.03$) between pre injury and 1wk post SCI. Abbreviations: V_{dep} = PCr depletion rate at onset of exercise; [Pi] = inorganic phosphate; [PCr] = phosphocreatine; [ADP]= cystolic adenosine triphosphate; Delta [PCr] = resting [PCr] – end exercise [PCr]; k_{PCr} = rate constant; 1/k = time constant; $V_{max\text{-}lin}$ = mitochondrial oxidative capacity based on recovery rate constant; V_{meas} = mitochondrial oxidative capacity based on

initial recovery rates; V_{ex} = mitochondrial oxidative capacity extrapolated from rate constant and delta PCr; $Q_{max\text{-}ADP}$ = maximum oxidative ATP synthesis rates based on k_{PCr} and [ADP]; $Q_{max\text{-}ADP*Pi/ATP}$ = oxidative ATP synthesis rates based on kPCr and phosphorylation potential.

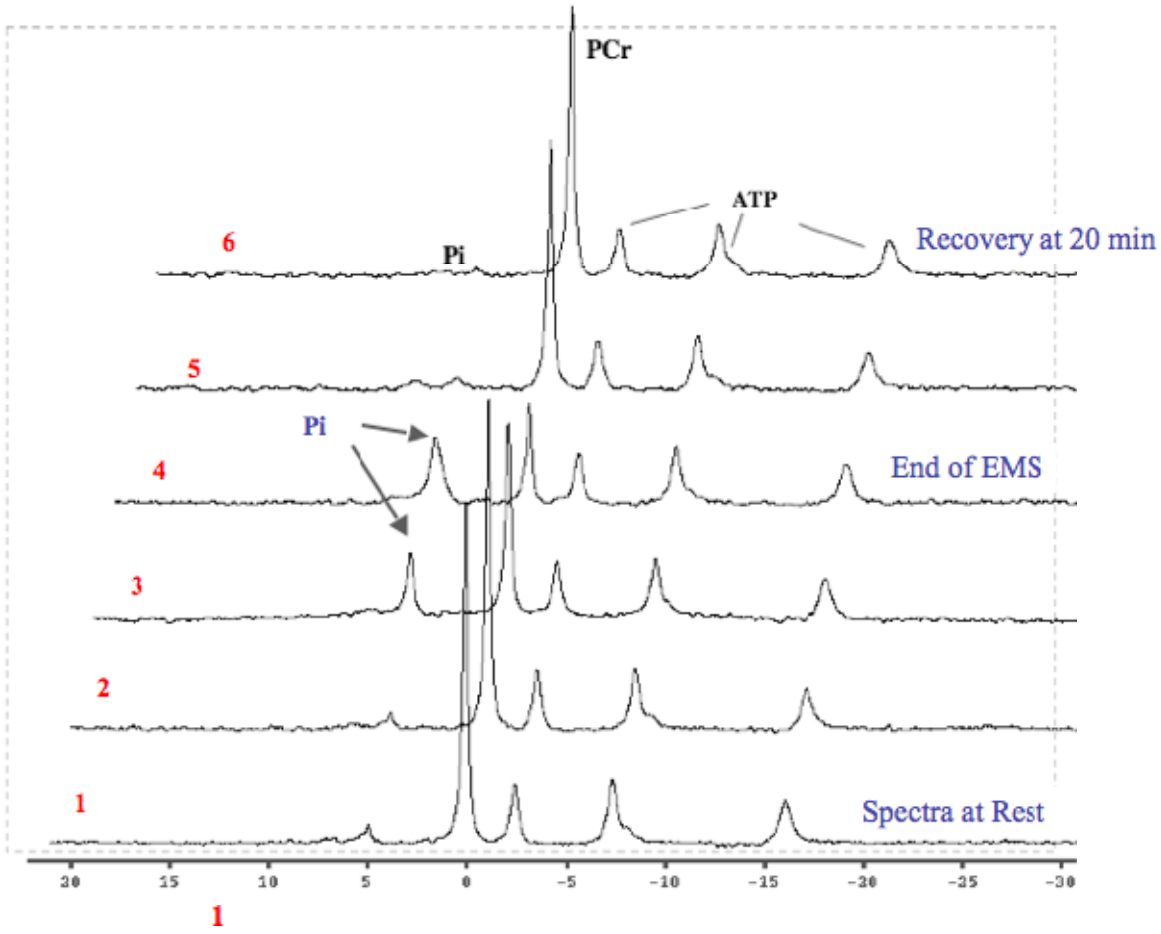

Figure 11-1. Representative kinetic ^{31}P spectra of rat gastrocnemius muscle at 11T. Selective spectra are shown from real-time in-vivo data obtained at rest (1,2), during electrical stimulation (EMS) (3,4) and during recovery (5,6). Note the elevation in Pi at end of EMS with concurrent decreases in PCr.

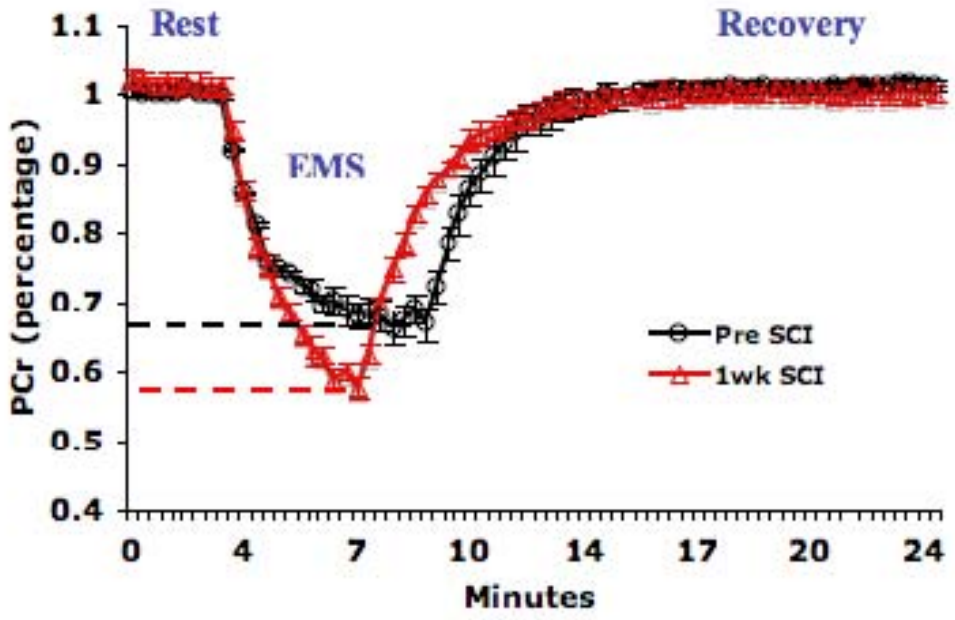

Figure 11-2. Average PCr depletion and recovery graphs in response to the stimulation protocol. Kinetic data during rest, electrical muscle stimulation (EMS) and recovery are compared before and after one week of spinal cord contusion in eight rats.

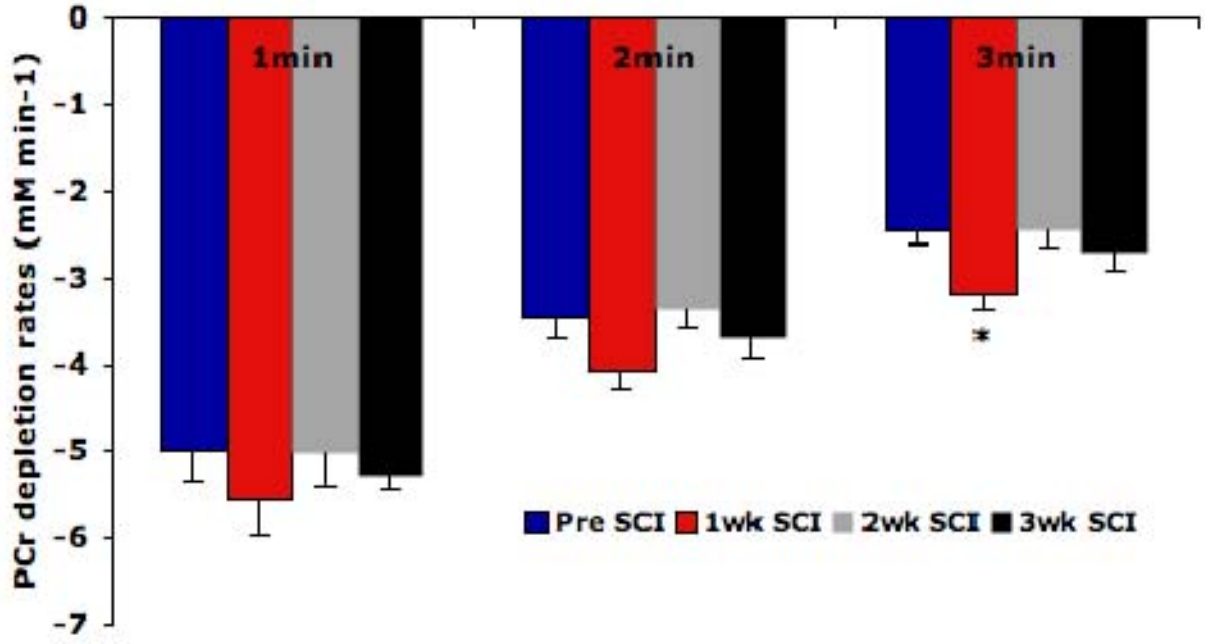

Figure 11-3. PCr depletion rates (1, 2, and 3min) before and after spinal cord contusion injuries (SCI). *Significant differences in three-minute (3min) depletion rates are seen between pre injury and 1week SCI.

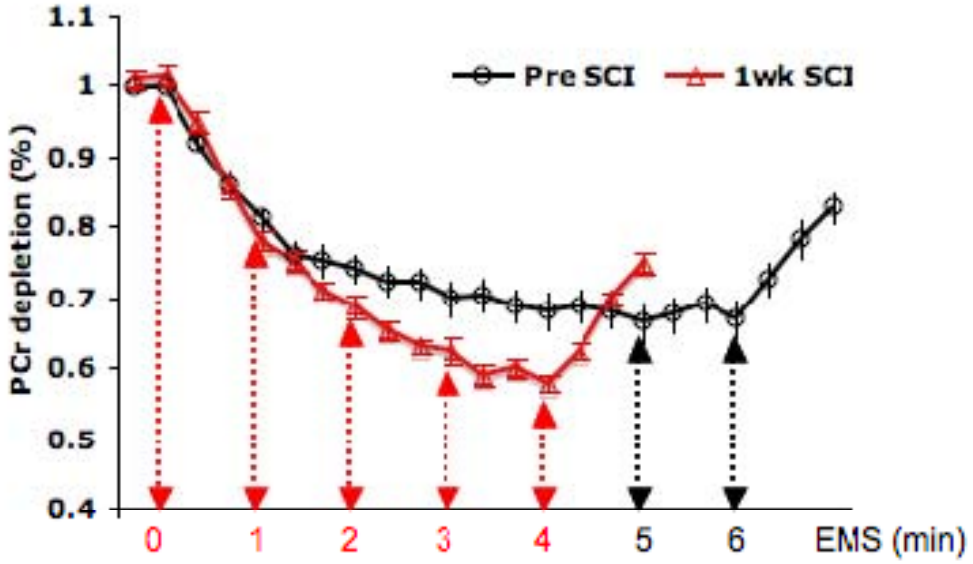

Figure 11-4. Expanded view of PCr kinetic data obtained at the onset of electrical muscle stimulation (EMS). PCr depletes earlier and farther after one week of spinal cord contusion (SCI) than before injury.

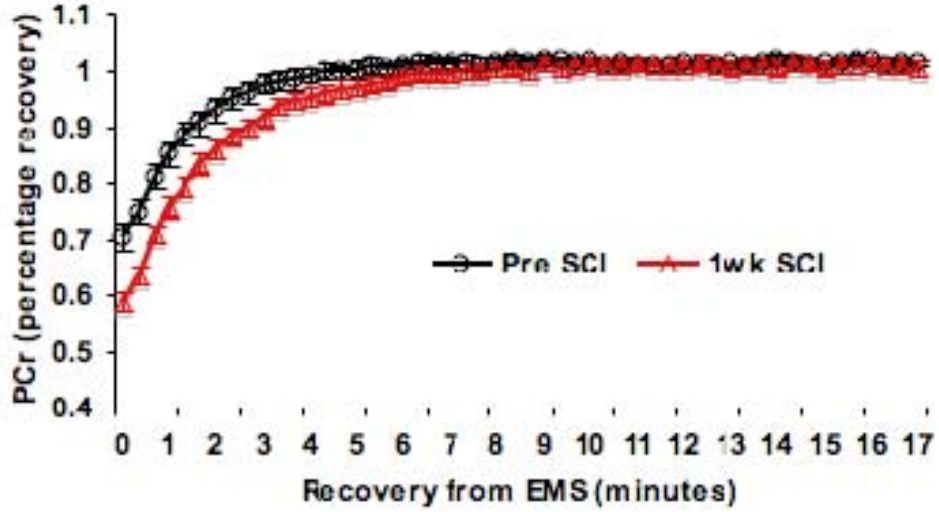

Figure 11-5. PCr recovery graphs obtained immediately after termination of electrical muscle stimulation (EMS). k_{PCr} are obtained after fitting the recovery graphs to a single monoexponential function. Note that end stimulation PCr content is different before and after injury.

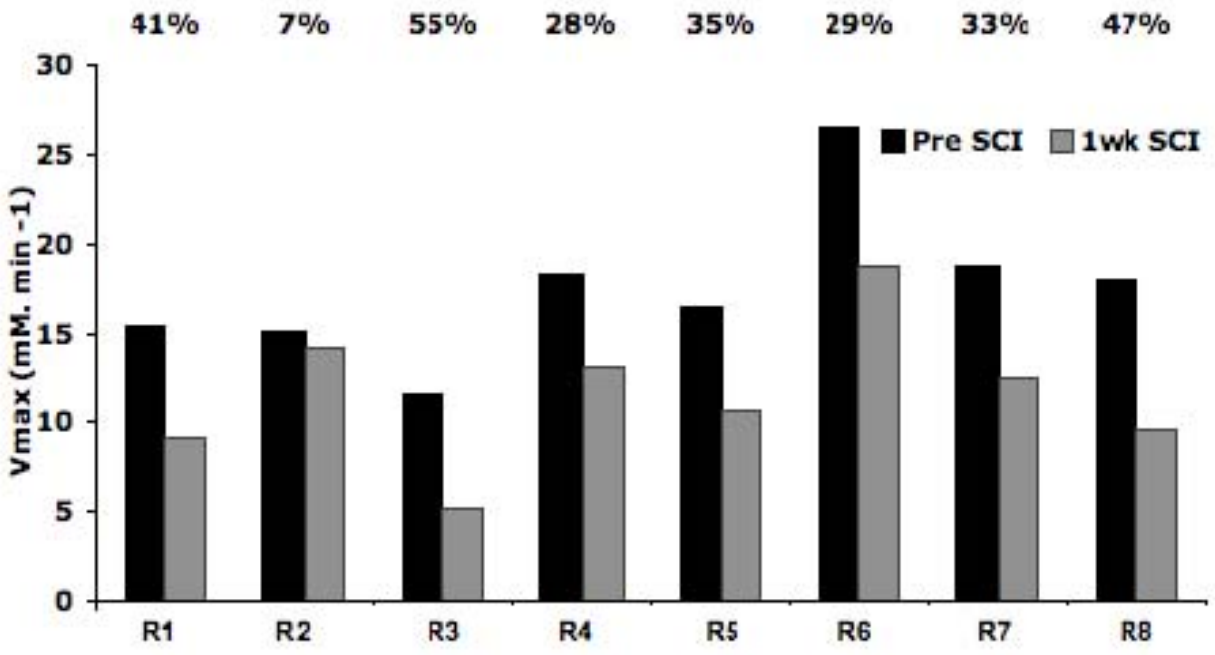

Figure 11-6. Maximum mitochondrial oxidative capacity (V_{max} - $mM.min^{-1}$) data from eight rats (R1-R8). Percentages indicate declines in V_{max} values after one week of spinal cord injury.

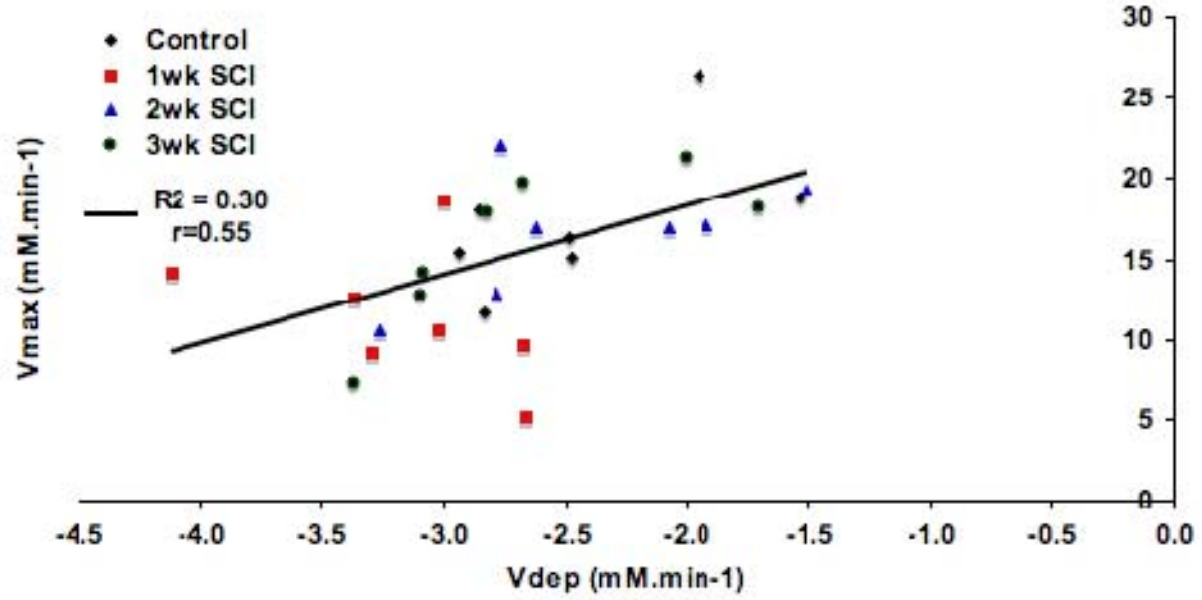

Figure 11-7. Scatter-plot depicting relationship between maximum mitochondrial capacity (Vmax) and rate of PCr depletion (Vdep). Data of all animals is pooled from each time point (before SCI and 1, 2, and 3 weeks post injury).

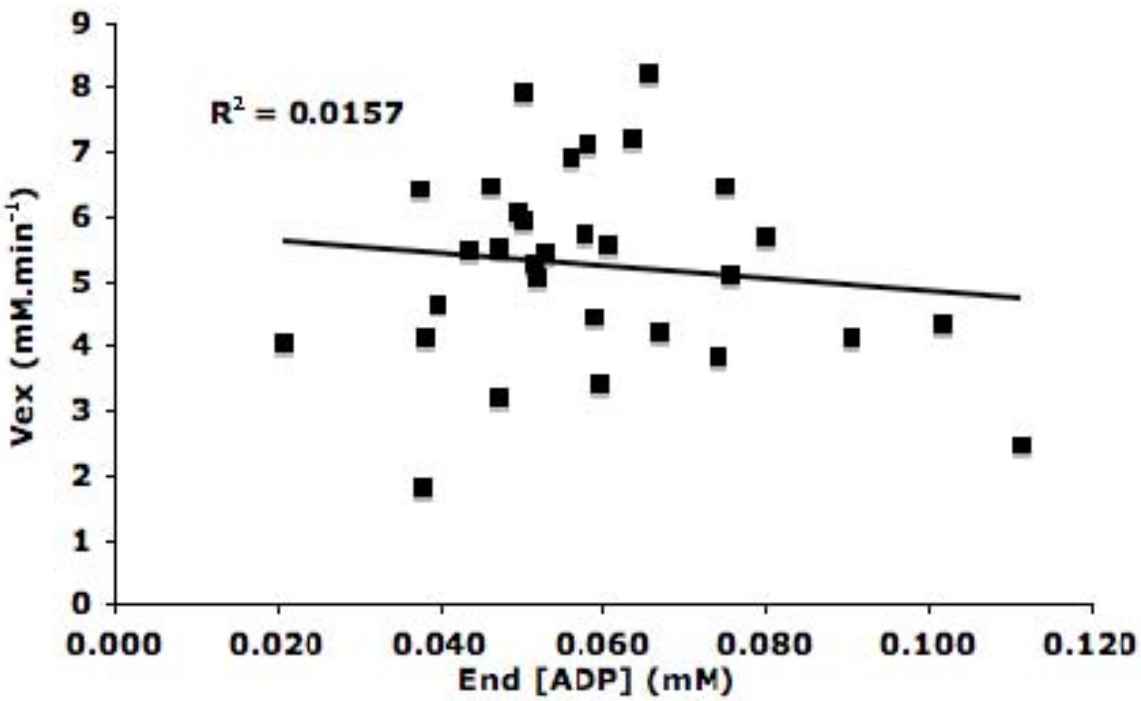

Figure 11-8. Relationship between end stimulation [ADP] and initial rates of PCr recovery (V_{ex}). Data of all animals is pooled from each time point (before SCI and 1, 2, and 3 weeks post injury).

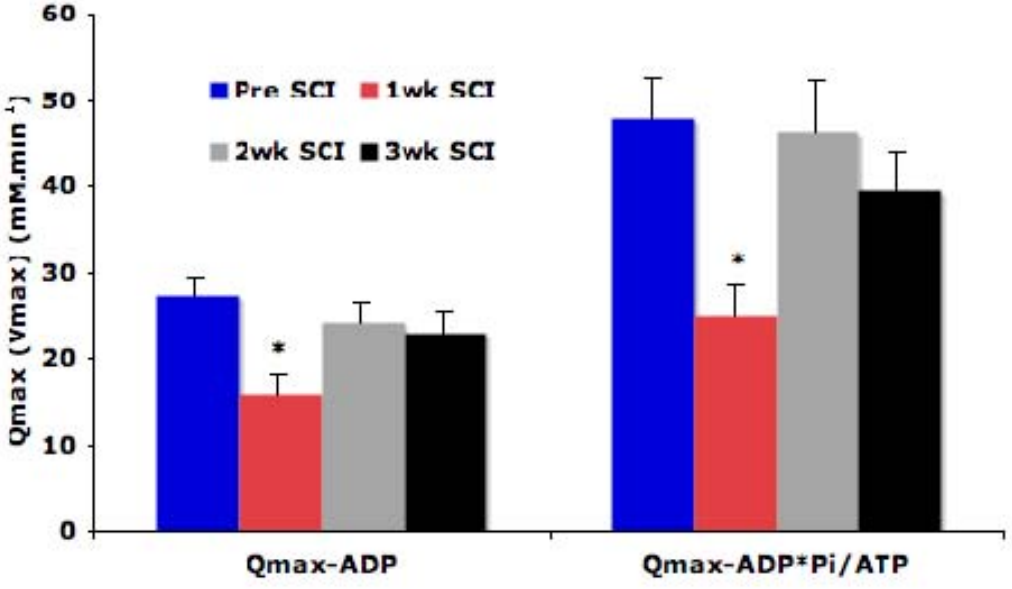

Figure 11-9. Maximal rates of oxidative capacity (Q_{max}) based on the [ADP] and [ADP][Pi]/[ATP] models. *Significant declines are seen in the injured group at two weeks after injury.

CHAPTER 12
CONCLUSION

Studying muscle adaptations in persons with incomplete SCI has been challenging because of the "incomplete" nature of the injury and heterogeneity of this patient group. This dissertation work demonstrates that despite the presence of relatively greater activity levels in persons with incomplete spinal cord injuries (SCI), lower extremity muscles below the site of lesion exhibit considerable motor impairments. Data from human studies in this work illustrate that the incompletely paralyzed muscle displays marked muscle atrophy, significant fatty tissue infiltration and a predisposition to muscle injury. These alterations are partially reversible by physiologically based therapeutic interventions such as locomotor training. Similarly, data from the animal studies demonstrate disturbances in the phosphorylation potential states and marked alterations in the *in-vivo* mitochondrial metabolic capacity of the skeletal muscle after spinal cord contusion in rats. An understanding of the underlying physiology of muscular adaptations can assist clinicians and therapists in strategizing specific therapeutic interventions for this patient cohort.

The present work will provide a foundation from which the relationship between skeletal muscle adaptations and function in this population can be further explored. Moreover, the use of sophisticated MR techniques will enable characterizing the paralyzed muscle non-invasively and with high resolution; while also allowing longitudinal follow-ups - all of which are crucial in assessing injury mechanisms, disease progression and efficacy of therapeutic interventions.

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BIOGRAPHICAL SKETCH

Prithvi Shah was born in August 1978, in a small town of Gujarat state in India. At the age of five years, Prithvi's parents sent her to a boarding school in western India. With a burning desire to award the best education to their daughters and inculcate in them good English speaking and writing skills, Mr and Mrs Shah let out their three girls from home for an expedition of a lifetime.

Prithvi successfully completed her high school with distinction (equivalent to Honors in the US) from St.Joseph's Convent School in Panchgani, Maharashtra. Having made up her mind to have a career in the health sciences, she then attained her undergraduate degree in physical therapy from Pune, India. While in the rehabilitation setting, her training encompassed interactions with patients from diverse backgrounds. However, the fervor of understanding mechanisms relevant to disease processes and strategizing therapies for patients led her to the United States in pursuit of a higher education.

In her training as a doctoral student at the University of Florida, Prithvi has been a recipient of numerous awards including scholarships for international students, nomination for the best teaching assistant and research awards. Under the proficient tutelage of Dr. Krista Vandenborne, Prithvi has had the unique opportunity to master her skills in both human and animal research of spinal cord injury.

Her immediate short term goals vest in further exploring medical technology and learning a variety of research tools to assess basic physiological processes concerning functioning of the nervous system. Among the long term goals are pursuing an academic career. With a passion for teaching and research, an academic career seems the right choice to make.

Prithvi Shah has a publication record of eight research articles (with two as the first author) and looks forward to publications from her last two studies in the current dissertation.