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# Assessment of low-force exercise in human paralyzed muscle

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# ASSESSMENT OF LOW-FORCE EXERCISE IN HUMAN PARALYZED MUSCLE

by

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A thesis submitted in partial fulfillment

of the requirements for the Doctor of Philosophy

degree in Physical Rehabilitation Science in the

Graduate College of

The University of Iowa

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Thesis Supervisor: Professor Richard K. Shields

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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee for
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# ABSTRACT

The loss of physical activity after a spinal cord injury results in musculoskeletal deterioration and metabolic dysfunction. Rehabilitation often overlooks the importance of physical activity in paralyzed limbs for systemic metabolic health. There is a need for safe feasible exercise interventions to increase physical activity levels for people with chronic paralysis and severe musculoskeletal loss. The goal of this work was to 1) develop a gene expression signature after a single dose and long-term training with high-force exercise using neuromuscular electrical stimulation for people with a recent spinal cord injury; 2) develop a novel low-force exercise intervention using neuromuscular electrical stimulation to increase routine physical activity and challenge chronically paralyzed human skeletal muscle; 3) determine the gene expression signature after a single dose of low-force exercise in people with long-term paralysis; and 4) develop an estimate for the dose of low-force exercise needed to transition chronically paralyzed muscle to a more metabolic and fatigue-resistant phenotype.

The major findings of this research were 1) genes with increased expression after long-term training are consistent with a healthy metabolic phenotype, while a single dose of exercise increased the expression of key transcription factors in people with paralysis; 2) low-force exercise limited force production and challenged chronically paralyzed muscle; 3) a single dose of low-force exercise increased the expression of key regulatory genes needed to improve muscle health; and 4) future work should utilize 90-minute sessions of low-force exercise performed 5 times a week to optimize physiologic changes in chronically paralyzed muscle. Together, this work supports the use of a low-force exercise intervention for people with long-term spinal cord injury and establishes the need for future work assessing systemic effects of low-force exercise on the health and quality of life for people with spinal cord injury.

# PUBLIC ABSTRACT

Routine physical activity promotes health and reduces the risk of developing some chronic diseases. People with a spinal cord injury are unable to move their paralyzed limbs. Consequently, muscle and underlying bone begin to deteriorate. Muscle has an important role regulating blood sugar levels. The loss of muscle after a spinal cord injury impairs the body's ability to regulate blood sugar, contributing to the development of chronic diseases like type 2 diabetes. The goal of this research was to develop a safe, feasible, and efficacious exercise intervention using neuromuscular electrical stimulation for with a spinal cord injury. We developed a novel low-force exercise using neuromuscular electrical stimulation to evoke muscle contractions in people with long-term paralysis. We tested the local effect of our low-force exercise on one limb of people with paralysis while keeping the other limb as a control. Our low-force exercise challenged paralyzed muscle and initiated key changes in gene expression needed to restore muscle's ability to contribute to blood sugar regulation. The effect of our low-force exercise was maximized when performed for 90-minutes at least 5 times per week. Together, these results regarding local skeletal muscle changes support the use of a low-force exercise in people with chronic spinal cord injury. Future studies using multiple muscle groups are needed to determine how low-force exercise influences the health, quality of life, and blood sugar regulation of people with long-term spinal cord injury.

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# CHAPTER 1 INTRODUCTION

Routine physical activity promotes health; however, the physical activity level of people with a spinal cord injury is often overlooked in rehabilitation. Physical inactivity contributes to the development of metabolic instability (Banerjea et al. 2008), particularly for people with long-term spinal cord injury. Existing exercise guidelines (Evans et al. 2015; Jacobs and Nash 2004) are not consistently implemented in rehabilitation clinics, in part, due to barriers like physical limitations, environment, lack of perceived benefit, and cost (Williams et al. 2014). There is a need to alleviate these barriers using efficacious, cost-effective, safe, and feasible exercise interventions, particularly for people with long-term paralysis who have significant musculoskeletal deterioration. We developed a novel low-force lower extremity exercise using neuromuscular electrical stimulation to increase the physical activity level for people with long-term paralysis. The goal for this research was to determine the localized effects of low-force exercise in chronically paralyzed skeletal muscle. The results of this work will allow future studies to determine how low-force exercise training affects the health and quality of life for people with long-term spinal cord injury.

## Background

### *Skeletal Muscle as a Systemic Metabolic Regulator*

Skeletal muscle is an adaptive tissue with a primary role of torque production during movements. The unique arrangement of sarcomeres in skeletal muscle coupled with nervous system control gives us a wide range of movement. Each movement uses a series of contraction and relaxation cycles in muscle fibers working in tandem to develop torque and implement a movement (Lieber 2002). Muscle contractions and subsequent

relaxations are active processes, requiring energy. Adenosine triphosphate (ATP) is the muscle's energy source, created through the breakdown of sugars, fats, and proteins. Healthy, active skeletal muscles are primary consumers of sugar in the body, making healthy skeletal muscle key for effective blood sugar regulation (Egan and Zierath 2013; Zierath and Hawley 2004).

Human skeletal muscle evolved to be highly responsive to bouts of physical activity. Routine physical activity is needed to maintain the contractile (sarcomere) and metabolic (mitochondrion) machinery in muscle. The sarcomere uses ATP to generate toque during a muscle contraction(Huxley 1969; Lieber 2002); while the enzymes of glycolysis, in the cytosol, and tricarboxylic acid cycle (TCA), in the mitochondria, replenish ATP by metabolizing substrates like glucose (Lieber 2002). Healthy, active muscles have well-developed mitochondria; while inactive muscles have less developed mitochondria (Pette and Spamer 1986; Shields 1995). The lack of well-developed mitochondria limits the metabolic capacity of inactive muscle, compromising glucose metabolism and the muscle's need for glucose from the blood (Kim et al. 2008; Stuart et al. 2013a). When active muscle suddenly becomes inactive after a spinal cord injury, an adaptive response is triggered resulting in a transformation from a metabolically healthy muscle to that of inactive muscle phenotype with a limited metabolic capacity (Dudley-Javoroski and Shields 2008b; Pette 2002; Shields 1995). Ultimately, this transformation disrupts blood glucose regulation and underscores the importance of routine physical activity for people with spinal cord injury.

Traditional methods of classifying the phenotype of skeletal muscle are invasive using tissue slices and protein staining. Muscle potentiation and fatigue are used as non-invasive descriptors for muscle phenotypes (Alway et al. 1987; Edwards et al. 1977;

Thorstensson and Karlsson 1976; Vandervoort and McComas 1983). Potentiation describes the increased force generated by a muscle during a contraction after previous activity. In contrast, fatigue describes the loss in force generated during a contraction from previous repeated bouts of activity. Muscle fibers with well-developed mitochondria potentiate slowly and experience less fatigue compared to muscle fibers fast-twitch muscle fibers with poorly developed mitochondria (Joseph et al. 2006; Shields 1995).

Muscle potentiation results from the interaction of sarcomere proteins with calcium ions. During a muscle contraction, calcium is released into the cytosol from the sarcoplasmic reticulum. In the cytosol, calcium interacts with the sarcomere proteins myosin regulatory light chain (RLC) and myosin light chain kinase (MLCK) (Rassier 2000; Rassier and Macintosh 2000). RLC is a protein complex that regulates the binding of myosin with actin, the force generating proteins. MLCK is a protein complex that phosphorylates RLC in the presence of calcium. The release of calcium during successive muscle contractions increases calcium concentrations and activates MLCK. Activated MLCK phosphorylates RLC, which increases RLC calcium sensitivity and subsequent force developed during the muscle contraction (MacIntosh et al. 2012; Moore and Stull 1984; Tubman et al. 1996; Tubman et al. 1997; Vandenboom and Houston 1996).

In competition with potentiation is muscle fatigue. Two forms of fatigue occur during evoked contractions, low-frequency fatigue (LFF) and high-frequency fatigue (HFF). HFF is a disruption in the transmission of a nerve stimulus before or at the neuromuscular junction. HFF occurs when high stimulation frequencies (>80Hz) are used and the time between stimulus pulses limits the nerve's ability to release, sequester, and re-release acetylcholine within the neuromuscular junction (Edwards et al. 1977). HFF is short lasting and can be prevented by using lower stimulation frequencies. LFF occurs during

prolonged muscle activity using low stimulation frequencies (<40Hz) and is predominantly due to the disruption of excitation-contraction coupling (Duchateau et al. 1987; Edwards et al. 1977; Eisenberg and Gilai 1979). Excitation-contraction coupling is the process by which an electrical action potential is transduced into a physical contraction and force production. Two proteins, ryanodine (RyR) and dihydropyridine (DHPR) sense the electrical muscle membrane potential along the sarcolemma and release calcium from the sarcoplasmic reticulum, eliciting muscle contractions from sarcomeres. LFF occurs when elevated adenosine diphosphate (ADP) and adenosine monophosphate (AMP) phosphorylates RyR (Duchateau et al. 1987; Eisenberg and Gilai 1979; Fauler et al. 2012; MacIntosh et al. 2012). Phosphorylated RyR has a decreased sensitivity to calcium becoming less responsive to subsequent action potentials (Duchateau et al. 1987; Eisenberg and Gilai 1979; Fauler et al. 2012; MacIntosh et al. 2012). Muscles with well-developed mitochondria are less susceptible to LFF, in part, because they limit elevated ADP and AMP concentrations through the metabolic synthesis of ATP (Jacobs et al. 2013). Inactive muscles have fewer mitochondria; consequently, inactive muscles are highly susceptible to LFF (Stephenson and Hawley 2013; Zierath and Hawley 2004).

*Genomic Regulation of Skeletal Muscle Phenotypes*

Muscle phenotypes help to describe the contractile and metabolic proteins being synthesized within a muscle fiber. Proteins are created through the transcription and subsequent translation of specific genes. Genes are small segments of deoxyribonucleic acid (DNA) that encode the sequence of amino acids needed to produce a protein. DNA is transcribed into messenger ribonucleic acid (mRNA) in the nucleus, transported to a ribosome in the cytosol, and translated into a protein. During translation, an amino acid

chain is constructed using the mRNA transcript as a template to create a functional protein.

The determination of which genes to transcribe (gene expression) and translate into functional proteins is dependent on the needs of the muscle fiber. Although specific mechanisms are often still unknown, physiologic stressors, like physical activity, are potent initiators of regulatory pathways for mRNA transcription and protein synthesis (Goldspink 2003; Mahoney et al. 2005a; Mootha et al. 2003). For example, routine physical activity increases the resting expression of peroxisome proliferator-activated receptor gamma coactivator receptor 1 alpha (PGC-1α) (Adams et al. 2011; Alvehus et al. 2014; Chan and Arany 2014; Mootha et al. 2003) and maintains a fatigue resistant phenotype. Routine physical activity also decreases the expression of myostatin (MSTN), which is an initiator of muscle atrophy (Adams et al. 2011; Rodriguez et al. 2014). Other genes like nuclear receptor 4 subfamily A 3 (NR4A3) (Nader et al. 2014; Pearen et al. 2012) and actin binding rho activating protein (ABRA) (Wallace et al. 2011) are acutely responsive to bouts of physical activity. Continued expression of these early response genes is important for skeletal muscle adaption (Joseph et al. 2006). Because the transformation of skeletal muscle from a fatigable phenotype to a fatigue resistant phenotype is a slow process, quantifying immediate expression responses can help predict potential long-term adaptions of an intervention prior completing expensive training trials.

*Neuromuscular Electrical Stimulation as an Exercise Intervention*

Neuromuscular electrical stimulation is a common intervention used to evoke a muscle contraction by stimulating motor neurons without central nervous system involvement. Neuromuscular electrical stimulation uses an electric current to depolarize a motor

neuron. Subsequently, the motor neuron activates a muscle fiber causing a muscle contraction. Two parameters can define a neuromuscular electrical stimulation protocol: stimulus intensity and stimulus frequency. The stimulus intensity is the current delivered during a stimulus pulse; and the stimulus frequency defines the time given between multiple stimulus pulses.

A group of muscle fibers with the activating motor neuron defines the motor unit. Motor units vary in size, phenotype, and recruitment order during a volitional contraction (Henneman 1957; Henneman et al. 1965; Olson et al. 1968). Small motor units are a collection of a few fatigue resistant muscle fibers with a small diameter motor neuron. In contrast, large motor units have large numbers of more fatigable muscle fibers paired with a large diameter motor neuron (Burke et al. 1973). During volitional contractions, motor units are recruited from small to large based on the required level of torque or speed for the contraction (Henneman 1957; Olson et al. 1968). Unlike volitional contractions, evoked contractions using neuromuscular electrical stimulation recruit motor units in a large to small pattern based on stimulus intensity (Bickel et al. 2011). Low stimulus intensities recruit larger more fatigable motor units, while high stimulus intensities recruit the smaller fatigue resistant motor units in addition to the larger motor units (Bickel et al. 2011).

The stimulus frequency determines the contraction force during evoked contractions. To elicit high-force contractions, stimulus pulses are delivered at high frequencies (>15Hz). These high frequency trains use the force summation properties of muscle to summate the force generated after each stimulus pulse. Consequently, low stimulation frequencies (<5Hz) yield low-force, twitch-like contractions (Cooper 1930). Unique to neuromuscular

electrical stimulation is the capacity to recruit of nearly all muscle fibers regardless of the stimulus frequency and resulting contraction force.

In people with paralysis, neuromuscular electrical stimulation is used as a substitute for the central nervous system. Usually paralyzed limbs experience little to no physical activity during the day. Physical activity encompasses any movement of skeletal muscle that results in a change in energy expenditure (Caspersen et al. 1985). Exercise defines a planned, structured use of physical activity to improve physical fitness and health (Caspersen et al. 1985). Consequently, the planned and structured use of neuromuscular electrical stimulation training constitutes an exercise intervention for people with spinal cord injury. Because of severe musculoskeletal deterioration, exercise interventions for people with spinal cord injury should be implemented cautiously, particularly when using parameters that result with high-force muscle contractions.

## Purpose and Significance

A spinal cord injury wreaks havoc on body systems, triggering a state of metabolic chaos, musculoskeletal deterioration, and other serious secondary complications (Dudley-Javoroski and Shields 2008b; 2012; Heiden 2013; McCully et al. 2011). Skeletal muscle transforms from a fatigue resistant to a fast fatigable phenotype, impairing a key blood glucose regulator (Bauman et al. 1999b; Duckworth et al. 1983; Duckworth et al. 1980; Dudley-Javoroski and Shields 2008b; Shields 1995). The underlying bone weakens, becoming severely osteoporotic and prone to fractures (Dudley-Javoroski and Shields 2008b; 2012; Edwards et al. 2013; Lala et al. 2013). In rehabilitation, the physical activity level of people with paralysis is often overlooked. Rehabilitation exercises using neuromuscular electrical stimulation to generate high-force muscle contractions increase physical activity levels and preserve the musculoskeletal system of

paralyzed limbs (Dudley-Javoroski and Shields 2008a; b; Petrie et al. 2014b; Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006b). However, unless these interventions are started early, high-force contractions would be inadvisable for people with long-term paralysis. There is a need to develop safe, inexpensive, and efficacious rehabilitation interventions for people with spinal cord injury.

## Specific Aims

Current rehabilitation practice poorly addresses the severe musculoskeletal deterioration after a spinal cord injury. Exercises using high-force muscle contractions evoked with neuromuscular electrical stimulation are available, yet they are not commonly used for long-term exercise training. Because these high-force contractions are not suitable for people with long-term spinal cord injury, we sought to develop and assess a new rehabilitation exercise intervention for people with chronic spinal cord injury.

*Specific Aim 1 (Chapter 2)*

Develop a gene expression signature in response to a high-force exercise using neuromuscular electrical stimulation in people with an acute spinal cord injury.

Specific Aim 1.1

Determine the gene expression signature after a single dose of a high-force exercise using neuromuscular electrical stimulation in people with an acute spinal cord injury

*Hypothesis 1.1 A single dose of high-force electrically stimulated muscle activity will increase the expression of metabolic transcription factors key for the transformation of muscle fibers from fast to slow.*

Specific Aim 1.2

Determine the gene expression signature after a long-term training with a high-force exercise using neuromuscular electrical stimulation in people with a spinal cord injury.

*Hypothesis 1.2 Long-term training with high-force electrically stimulated muscle activity will increase the expression of metabolic and contractile protein genes associated with slow, oxidative muscle fibers.*

*Specific Aim 2 (Chapter 3)*

Determine the effect a low-force exercise using neuromuscular electrical stimulation has on the muscle of people with and without a spinal cord injury.

Specific Aim 2.1

Determine the effect a low-force exercise using neuromuscular electrical stimulation on muscle fatigue in non-paralyzed and paralyzed human skeletal muscle.

*Hypothesis 2.1 Low-force exercise using electrical muscle activity will cause more muscle fatigue in paralyzed human skeletal muscle compared to non-paralyzed human skeletal muscle.*

Specific Aim 2.2

Determine the effect a low-force exercise using neuromuscular electrical stimulation on post-activation potentiation in non-paralyzed and paralyzed human skeletal muscle.

*Hypothesis 2.2 Low-force exercise using neuromuscular electrical stimulation will increase post-activation potentiation in chronically paralyzed human skeletal muscle compared to non-paralyzed human skeletal muscle.*

Secondary Aim 2.3

Explore the relationship between homeostatic gene expression of metabolic and contractile proteins in non-paralyzed and chronically paralyzed human skeletal muscle and muscle fatigue after a low-force exercise using neuromuscular electrical stimulation.

*Hypothesis 2.3 Chronically paralyzed human skeletal muscle will express genes consistent with a highly fatigable muscle fiber phenotype, while non-paralyzed human skeletal muscle will express genes consistent with a fatigue-resistant muscle fiber phenotype.*

*Specific Aim 3 (Chapter 4)*

Determine the effect a single dose of low-force exercise using neuromuscular electrical stimulation has on chronically paralyzed human skeletal muscle.

Specific Aim 3.1

Determine the fatigue and post-activation potentiation of 2 exercise protocols (5Hz and 20Hz) using neuromuscular electrical stimulation with chronically paralyzed human skeletal muscle.

*Hypothesis 3.1 A single dose of exercise using 5Hz neuromuscular electrical stimulation will fatigue chronically paralyzed human skeletal muscle more than a dose of exercise using 20Hz neuromuscular electrical stimulation.*

Specific Aim 3.2

Determine the effect 2 exercise protocols (5Hz and 20Hz) using neuromuscular electrical stimulation have on the expression signature of metabolic transcription factors in chronically paralyzed human skeletal muscle.

*Hypothesis 3.2 A single dose of exercise using 5Hz neuromuscular electrical stimulation will increase the expression of metabolic transcription factors more than a single dose of exercise using 20Hz neuromuscular electrical stimulation in chronically paralyzed human skeletal muscle.*

Specific Aim 3.3

Determine the effect 2 exercise protocols (5Hz and 20Hz) using neuromuscular electrical stimulation have on the expression of key contractile transcription factors in chronically paralyzed human skeletal muscle.

*Hypothesis 3.3 A single dose of exercise using 5Hz neuromuscular electrical stimulation will increase the gene expression of slow-twitch transcription factors more than a single dose of exercise using 20Hz neuromuscular electrical stimulation in chronically paralyzed human skeletal muscle.*

*Specific Aim 4 (Chapter 5)*

Explore how the dose of low-force exercise training using neuromuscular electrical stimulation influences physiologic biomarkers indicative of a phenotype transformation in chronically paralyzed human skeletal muscle.

Specific Aim 4.1

Explore the dose of low-force exercise using neuromuscular electrical stimulation needed to change fatigability of chronically paralyzed human skeletal muscle.

*Hypothesis 4.1 There will be a strong positive correlation between the training dose (number of training sessions per week) of a low-force exercise using neuromuscular electrical stimulation and change in fatigability of chronically paralyzed human skeletal muscle.*

Specific Aim 4.2

Explore the dose of low-force exercise using neuromuscular electrical stimulation needed to change the gene expression signature of chronically paralyzed human skeletal muscle.

*Hypothesis 4.2. There will be a strong positive correlation between the training dose (number of training sessions per week) of a low-force exercise using neuromuscular electrical stimulation and the change in expression of key genes indicative of a fatigue-resistant phenotype transformation in chronically paralyzed human skeletal muscle.*

Secondary Aim 4.3

Explore the relationship between the gene expression signature and the fatigability in chronically paralyzed human skeletal muscle after 12 weeks of low-force exercise training using neuromuscular electrical stimulation.

*Hypothesis 4.3. There will be a strong positive correlation between the change in fatigability and the expression of genes indicative of a transformation to a more fatigue resistant phenotype after low-force exercising training using neuromuscular electrical stimulation in people with chronic spinal cord injury.*

# CHAPTER 2 A MINIMAL DOSE OF ELECTRICALLY INDUCED MUSCLE ACTIVITY REGULATES DISTINCT GENE SIGNALING PATHWAYS IN HUMANS WITH SPINAL CORD INJURY

## Introduction

Muscle paralysis after a spinal cord injury triggers a cascade of events that disrupts the metabolic homeostasis of paralyzed muscle. Healthy skeletal muscle is involved with over 70% of daily glucose utilization (Bjornholm and Zierath 2005). Paralyzed muscle rapidly atrophies and transforms oxidative fibers into predominantly fast-twitch, glycolytic fibers (Crameri et al. 2002; Grimby et al. 1976; Petrie et al. 2014a; Shields 1995; Shields et al. 2006a; Shields and Dudley-Javoroski 2006; Shields et al. 1997). Skeletal muscle that becomes glycolytic is a precursor to decreased insulin receptor sensitivity (Stuart et al. 2013b). Individuals with spinal cord injury are at a higher risk of developing metabolic syndrome, diabetes, heart complications, and renal failure (Bauman et al. 1999a; Cragg et al. 2013; Duckworth et al. 1980; Jensen et al. 2012; Lavela et al. 2006). We have demonstrated that regular training of paralyzed muscle reduces muscle atrophy, preserves fatigue-resistance, and maintains the underlying skeletal system in people with spinal cord injury (Adams et al. 2011; Dudley-Javoroski et al. 2011; Shields and Dudley-Javoroski 2006; Zijdewind and Thomas 2012). However, we are not aware of the specific genes regulated by an acute bout of minimal muscle activity as compared to a long duration minimal muscle activity program in humans with paralysis.

Gene expression profiling is one method to survey the genome for mRNA transcripts common to a specific phenotype (Mootha et al. 2003). For example, through gene set

enrichment methods, diabetes was linked to a decrease in the expression of oxidative phosphorylation signaling pathways in skeletal muscle(Mootha et al. 2003). Unlike healthy people, who can ambulate throughout the day, people with paralysis experience limited muscle activity after the 10-30 minute bout of exercise using electrical stimulation. We are interested in understanding if a single dose of muscle activity (muscle activated for less than .6% of the day equal to ~ 10 minutes) versus a chronic dose of muscle activity (.6% of the day performed regularly over 1 year) regulates distinct gene transcription and metabolism pathways. No previous report, to our knowledge, has specifically determined if a short bout of muscle activity, induced electrically, regulates genes associated with glycolysis, tricarboxylic acid cycle (TCA), fatty acid oxidation, oxidative phosphorylation, and mitochondria dynamics (fission, fusion, and biogenesis); in particular, in contrast to the regulation induced through a long-term training period (> 1 year). .

Key muscle transcription factors and co-activators are known to be responsive to metabolic and mechanical stress induced through muscle contractions in healthy people (Egan and Zierath 2013). Previous studies have identified several of these stress response genes including peroxisome-proliferator-activated-receptor-gamma 1-coactivator-alpha (PGC-1α) (Egan et al. 2010; Kulkarni et al. 2012; Mootha et al. 2003; Wallace et al. 2011); nuclear orphan receptor-1 (NOR-1/NR4A3) (Kawasaki et al. 2009; Pearen et al. 2012); interferon-related developmental regulator-1 (IFRD1) (Micheli et al. 2011); and actin-binding Rho-activating protein (ABRA/STARS) (Lamon et al. 2009; Wallace et al. 2011; Wallace et al. 2012). The induction or repression of these major transcription factors would trigger a cascade of events ultimately leading to the transformation of the underlying metabolic state of paralyzed skeletal muscle. Accordingly, genes associated with glycolysis (PDK4, PDHA1, PDHB, and PDHX)

(Brown et al. 2004; Pilegaard et al. 2006; Pruitt et al. Oct. 9, 2002; Spriet and Heigenhauser 2002; Spriet et al. 2004; Sugden and Holness 2003), fatty acid oxidation (ACADVL, ACADL, ACAD8, ACAD9) (Andresen et al. 1996; Lea et al. 2000; Pruitt et al. Oct. 9, 2002; Strauss et al. 1995), tricarboxylic acid cycle (TCA) (MPC1, MPC2, OGDH, SDHB)(Bricker et al. 2012a; Pruitt et al. Oct. 9, 2002), oxidative phosphorylation (NDUFB1, NDUFA2, CYC1, COQ10A) (Gaignard et al. 2013; Pruitt et al. Oct. 9, 2002), and mitochondria dynamics including fission, fusion, and biogenesis (MFF, OPA1, MFN1, and MFN2) (Iqbal et al. 2013; Palikaras and Tavernarakis 2014; Pruitt et al. Oct. 9, 2002) and their associated pathways are the primary focus of this investigation.

The purpose of this study is to determine the effects of a single dose and a chronic dose of neuromuscular electrical stimulation on human paralyzed muscle. We expect a single dose of muscle activity will result in an increased expression of metabolic transcription factors associated with the transformation of fibers from fast to slow. In contrast, we expect the chronically trained muscle will show the long-term stable expression of genes associated with the maintenance of oxidative metabolic pathways, despite the minimal daily activity performed (10 minutes/day). Overall, these findings will support the hypothesis that a minimal dose of muscle activity, performed regularly, is an important and powerful metabolic regulator of skeletal muscle health, even in people with paralysis, who cannot exercise throughout the day.

## Methods

*Subjects*

Five males (30.40±4.39 years of age) with complete paraplegia participated in the current study. The protocol was approved by the University of Iowa Human Subjects

Review Board, and all subjects provided written informed consent before participating. Subjects received a single dose of neuromuscular electrical muscle stimulation unilaterally 3 hours before a bilateral soleus muscle biopsy (6 control and 6 activated limbs using a within subject control; 3 repeated as technical reference); or were muscle biopsied after training for > 1-year (4 control and 4 trained; 2 repeated as technical reference). All subjects had complete paraplegia (ASIA-A) at or below T4 and had been paralyzed for over 1.5 years. None of the subjects used neuromuscular electrical stimulation of the soleus prior to the start of this study. The training subjects were unique because they sustained over an 81% compliance with the training. Subjects discontinued training 5 days prior to the soleus muscle biopsy procedure to capture the homeostatic effect of long-term training and minimize the influence an acute dose of activity has on gene regulation.

*Acute Electrical Stimulation Protocol*

The acutely stimulated subjects had a single session of unilateral muscle stimulation of the soleus muscle. None of these individuals had received prior neuromuscular electrical stimulation. Subjects sat in their wheelchair while the experimenter positioned one leg, chosen randomly into the testing apparatus that stabilized the ankle to obtain an isometric contraction(Adams et al. 2011; Shields 1995; Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006c; Shields et al. 1997). The ankle and knee were flexed to 90º and the limb was secured to the apparatus with soft straps above the knee to provide resistance for an isometric contraction. The 90º knee angle minimizes the contribution of the gastrocnemius muscle to plantar flexion torque, creating a more reproducible measure of the performance of the soleus muscle (Shields 1995; Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006c; Shields et al. 1997).

Neuromuscular electrical stimulation was delivered to the plantar flexor muscles using reusable adhesive carbon electrodes. Stimulation was provided by a constant current electrical stimulator with a 0 to 400 milliamp range at 400 volts (Digitimer Model DS7A, Digitimer Ltd., Welwyn Garden City, Hertfordshire, England). The stimulator was controlled by digital pulses from a data-acquisition board (Metrabyte DAS 16F, Keithley Instruments Inc., Cleveland, Ohio, USA) housed in a microcomputer under custom software control. Single pulses were given at increasing intensity until maximal twitch torque was observed via an oscilloscope. Stimulation intensity was increased an additional 50% and remained at this level for the remainder of the experiment to insure supra maximal activation. Subjects performed 5 warm-up contractions (10 Hz, 7 pulses per contraction) to potentiate the plantar flexor muscles and minimize the risk of muscle strain. After the warm-up contractions, the plantar flexor muscles were activated with 10 Hz stimulation trains (7 pulses) every 2100ms for 120 isometric contractions. After 5 minutes of rest, subjects performed a second bout of 120 contractions at the same intensity and frequency. After the second bout, an experimenter used an indelible ink pen to mark the trained leg with the time that stimulation ended in preparation for the 3hr post-activation bilateral soleus biopsy. The biopsy sampled fibers that had received the acute dose of electrical stimulation.

*Chronic Electrical Stimulation Protocol*

The right soleus muscle was trained for at least one year then discontinued training 5 days prior to obtaining bilateral muscle biopsies. The training protocol consisted of 4 bouts of 125 contractions (15 Hz; train duration 667ms) per day with 5 minutes of rest between each bout (Adams et al. 2011; Shields and Dudley-Javoroski 2006). The subjects trained one limb while the opposite limb did not receive any electrical stimulation training. The subjects performed a total of 333.3 seconds of supra maximal

muscle activation per day, representing less than 0.6 % of the day that the muscle was active.

*MRI Acquisition and Analysis*

Due to the known association between metabolic risk and the fat to muscle ratio, we decided to illustrate the differences in phenotype between the trained and untrained limbs by using an axial-plane spin-echo, T1-weighted magnetic resonance (MR) image from the trained muscle and the untrained muscles of the lower leg and mid-thigh (1.5T Siemens Avanto Scanner). A three-dimensional gradient-echo-based sequence was used for high-resolution imaging. The acquisition parameters included repetition time (TR) of 15.0ms and echo-time (TE) of 6.7ms with a 512 x 256 matrix covering a field of view of 46 cm x 18 cm and a 2.5-mm slice distance, creating a voxel volume of about 1.58 $mm^3$. An MR was only obtained from subject 2, as subject 1 had metal fragments retained within his body from his initial injury (Adams et al. 2011). The MR images were analyzed using a custom MATLAB© (The Mathworks, Inc, Natick, MA, USA v.2011) script, which utilized image registration and segmentation algorithms implemented in the MATITK toolkit(Chu and Hamarneh 2006). Using the image histogram, the images were segmented in to three tissue types (adipose, muscle, and compact bone/background). After segmentation, the number of voxels corresponding to each tissue type was totaled and multiplied by the voxel volume of 1.58 $mm^3$, providing an estimate to the total area for each tissue type within the limb. The trained and untrained limbs were separated into two regional sites (the distal thigh and the proximal leg) each consisting of 30 image slices. The proximal leg maximized the inclusion of the soleus muscle, while the distal thigh was used as a control to illustrate the specificity of training to the soleus muscle. The total muscle, fat, and background volumes were compared between the trained and

untrained sides. The total muscle and fat tissue content within each region was totaled and a ratio of muscle to fat volume was calculated.

*Muscle Biopsy and Exon Microarray Procedure*

The biopsy procedure has been previously described (Adams et al. 2011). Briefly, percutaneous muscle biopsies were taken from both the intervention soleus muscle and the control soleus muscle of each subject using a Temno biopsy needle (T1420, CardinalHealth) under ultrasound guidance within a sterile field. Both limbs were biopsied during at the same time of day. Four passes of the needle were performed to obtain a wide sampling range within the muscle but limit injury to the patient for safety concerns. Following harvest, muscle biopsy samples were immediately placed In RNALater (Ambion) and stored at -80 °C until further use. Total RNA was extracted using TRIzol solution (Invitrogen) according to the manufacturer's instructions, as described previously (Adams et al. 2011). Microarray hybridizations were performed at the University of Iowa DNA Facility. Briefly, 50 ng of RNA was converted to SPIA amplified cDNA using the WT-Ovation Pico RNA Amplification System, v1 (NuGEN Technologies, San Carlos, CA, Cat. #3300) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN MinElute Reaction Cleanup column (QIAGEN Cat #28204) according to modifications from NuGEN. Four µg of SPIA amplified DNA were used to generate ST-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies, Cat #2000) and again cleaned up with the Qiagen column as above. 5µg of this product were fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module, v2 (NuGEN Technologies, Cat. #4200) per the manufacturer's recommended protocol. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Human

Exon 1.0 ST arrays (Part No. 900650), and incubated at 45º C for 18 h with 60 rpm rotation in an Affymetrix Model 640 GeneChip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), signal amplified with antistreptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade and data were collected using the GeneChip operating software (GCOS) v1.4.

*Exon Array Data Analysis*

The Affymetrix Human Exon 1.0 ST arrays were normalized using a Robust Multi-array Average (RMA) and transformed into a log2 hybridization signal, reflecting the mean signal intensity of all exon probes specific for a particular mRNA transcript using Partek Genomic Suites (v6.6 Copyright © 2013 Partek Inc., St. Louis, MO, USA). The mRNA log2 hybridization signals were further analyzed using two techniques: a pathway analysis system and expression level profiling.

The pathway analysis was performed using the gene set enrichment analysis (GSEA) algorithm implemented in Partek Genomic Suites (v6.6 Copyright © 2013 Partek Inc., St. Louis, MO, USA)(Subramanian et al. 2005). The Gene Ontology (GO) biological process database (version 3.1) was used to cluster differentially expressed gene transcripts to determine whether a particular pathway was likely up or down regulated in the acute and chronic groups. A false-detection rate (FDR) of 10%, lower than the recommended 25%, was used to determine those pathways significantly up or down regulated due to the small sample size.

The exon arrays were analyzed using an expression profiling technique, which compared the mRNA hybridization signals between the acute and chronic groups relative to each participant's control leg. MRNA with a log2 hybridization signal less than 2 standard deviations away from the mean hybridization signal were discarded from the dataset, restricting the analysis to mRNA transcripts with high signal intensity relative to background intensities. A fold-change (FC) was calculated for each gene transcript in the acute and chronic groups relative to the control limb. A gene transcript was considered differentially expressed, if 1) the FC was greater than 2.0 or less than 0.5 (indicating a 2.0 fold decrease in expression) and 2) the p-value for the gene was below 0.05 for an independent paired t-test comparing the stimulated and control. Due to the small sample size within each group, two muscles samples were independently extracted and hybridized for each subject, providing a technical replicate to minimize systematic errors.

*qPCR Procedure and Analysis*

Muscle samples were homogenized in lysis buffer using a tissue homogenizer and hard tissue grinding tip (Omni). A column-based RNA extraction was subsequently performed using the RNEasy Fibrous Tissue Kit (Qiagen). DNAse was included in the protocol to ensure absence of genomic DNA in final samples. RNA samples were eluted in water and quantified via nanodrop method. In addition, the quality of each sample was assayed using the Agilent 2100 Bioanalyzer. High quality RNA samples were reverse transcribed using iScript supermix (Bio-Rad). Input quantity of RNA was standardized across all reactions (500 ng each). All cDNA samples were stored at -86°C. At the time of qPCR plate preparation, cDNA samples were diluted five-fold in water and analyzed via SYBR green technology using a custom PrimePCR plate (Bio-Rad). All samples were analyzed in duplicate at a final concentration of .5 ng/μL cDNA per 10μL reaction

using an ABI 7900 machine. Expression levels were converted to a relative fold change (FC) of the acutely stimulated or chronically trained limb to the control limb using the comparative $C_T$ method described by Schmittgen and Livak (Schmittgen and Livak 2008). Beta 2 macroglobulin (B2M) was used as the reference gene because it demonstrated a consistent expression across all limbs.

## Results

*Descriptive Analysis of Training Phenotype*

The chronically paralyzed muscle phenotype is highly fatigable, slows its contractile speed during repetitive activation, and potentiates early during electrical stimulation, findings that are consistent with fast fatigable glycolytic muscle (Figure 2.1A). The effect of long-term training caused an increase in the lean muscle mass to adipose tissue ratio when compared to the untrained limb or to the upper thigh area that was not stimulated (Figure 2.1B). The muscle hypertrophy following long-term training and the reduced muscle fat are evident using MRI imaging from the trained and untrained limb of the same subject (Figure 2.1C and D). Note the increased green marker within the atrophied untrained muscle indicating an increase of intramuscular fat. The chronic training phenotype also showed less collagen IV (Figure 2.1E) and an increase in mitochondria signaling (Figure 2.1G) when compared to the same subject's untrained control limb (Figure 2.1F and H). The fatigue and contractile properties of the long-term trained soleus muscle have been previously reported (Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006c).

*Gene Enrichment Signaling Pathways*

We measured over 16,000 mRNA transcripts from each muscle sample obtained. Using a conservative false discovery rate (FDR) of 10% and the gene ontology (GO) biological process database, we identified 117 and 35 pathways that were significantly up regulated after an acute dose and chronic dose of electrical stimulation, respectively (Figure 2.2). Both the acute and chronic skeletal muscle groups demonstrated an increase in the expression of genes categorized as metabolic pathways; however, the genes with increased signal intensity differed between groups. An acute dose of electrical stimulation up regulated the expression of genes reported to be transcription factors and co-activators. In contrast, chronic training up regulated the expression of genes reported to be oxidative enzymes and metabolic transporter proteins. Many of the up regulated cell signaling and metabolic pathways involve the same genes (EIF4E, NR4A3, ABRA, EGR1, PGC-1A, MYL3, MYH7, TNNT1, and ATP2A2). EIF4E, NR4A3, ABRA, EGR1, and PGC-1A are transcription factors and co-activators important in maintaining oxidative muscle fibers. MYL3, MYH7, TNNT1, and ATP2A2 are contractile proteins observed in oxidative and not glycolytic muscle fibers.

*Individual Gene Analysis*

Over 16,000 mRNA transcripts had a high hybridization signal in both the acute and chronic groups. Of those, 104 genes had a 2-fold increase in expression and 0 genes had a 2-fold decrease in expression after a single session of neuromuscular electrical stimulation; however, after chronic stimulation training there were 66 genes with a 2-fold increase in expression, and 20 genes had a 2-fold decrease in expression. The top 10 genes with the highest relative fold change (Table 2.1 and Table 2.2) and lowest relative fold change (Table 2.3 and Table 2.4) are listed for the acute and chronic groups, respectively.

In the acute group, 8 of the top 10 high intensity transcripts function as transcription, translation, or enzymatic regulators (NR4A3, EGR1, FOS, GEM, ABRA, IFRD1, CYR61, and PGC-1α). PGC-1α, NR4A3, IFRD1, FOS, and ABRA trigger cellular processes needed to shift the muscle toward an oxidative phenotype, while CYR61 aids in initiating angiogenesis in muscle. PGC-1α, NR4A3, EGR1, IFRD1, FOS, and ABRA were over 2 fold higher following an acute bout of electrical stimulation in paralyzed muscle. In contrast, these genes were minimally altered in chronically trained paralyzed muscle. In the chronic group, 5 out of the 10 high intensity transcripts function as metabolic enzymes, protein transporters, and oxidative muscle proteins (MYH6, MYH7, MYL3, MYL2, and AGBL1). MYH6, MYL3, and MYH7 were at least 3 fold higher in chronically trained muscle relative to acutely stimulated paralyzed soleus muscle. Importantly, chronic training decreased genes associated with fast twitch muscle (ACTN3, MYLK2, and MYL5) and atrophy (MSTN) (Table 2.4). An acute session of electrical stimulation triggered a 1.8 fold decrease of MSTN (Table 2.3Table 2.3), and was the only common gene substantially decreased in both the acute and chronic groups.

*Transcription Factors and Muscle Fiber Types*

After a preliminary analysis, we looked at the influence of acute and chronic soleus stimulation on a subset of genes linked to skeletal muscle phenotypes or metabolic capacity, specifically targeting genes linked to skeletal muscle transcription factors, fast-twitch muscle proteins, slow-twitch muscle proteins, glycolysis enzymes, fatty acid oxidation enzymes, tricarboxylic acid cycle enzymes, oxidative phosphorylation enzymes, and mitochondrial fission and fusion proteins. PGC-1α(5.46±0.64, $p<0.001$), NR4A3 (12.45±2.36, $p<0.001$), and ABRA (5.98±0.40, $p<0.001$) are early response genes that were up regulated relative to the non-stimulated limb 3 hours after a single

bout of neuromuscular electrical stimulation. (Figure 2.3A-C) However, after chronic electrical stimulation training only PGC-1α (1.73± 0.09, p<0.002) was increased (Figure 2.3A). NR4A3 and ABRA demonstrated a relative decrease with chronic neuromuscular electrical stimulation training (Figure 2.3B and C). MSTN was decreased both 3 hours after a neuromuscular electrical stimulation exercise (0.56±0.06, p=0.002) and chronic training (0.33± 0.03, p<0.001) (Figure 2.3D). Genes linked to fast-twitch muscle fibers were decreased after chronic electrical stimulation training, but were, in general, unaltered 3-hours after a single session of neuromuscular electrical stimulation in chronically untrained paralyzed muscle (Figure 2.3E-H). In contrast, genes linked to slow-twitch muscle fibers were increased in chronically trained muscle, but decreased 3 hours after a single session of neuromuscular electrical stimulation (Figure 2.3I-L).

*Glycolysis and Fatty Acid Oxidation*

Glycolysis and fatty acid oxidation are the 2 primary pathways used to metabolize macromolecules in skeletal muscle. PDK4 was increased relative to the control limb 3 hours after a single session of neuromuscular electrical stimulation training (3.37±0.83, p=0.008), but was not increased with chronic muscle training (1.55±0.35, p=0.21) (Figure 2.4A). PDHA1 (1.60±0.057, p<0.001) PDHB (1.80±0.08, p<0.001), and PDHX (1.57±0.05, p<0.001) were all increased after chronic neuromuscular electrical stimulation training, but were not altered 3 hours after a single session of neuromuscular electrical stimulation (1.05±0.05, p=0.46, 1.11±0.09, p=0.35, 1.09±0.13, p=0.59; respectively) (Figure 2.4B-C). ACADVL (1.63±0.049, p=0.049), ACAD8 (1.33±0.089, p=0.023) and ACAD9 (1.16±0.023, p=0.006) were increased after chronic neuromuscular electrical stimulation training, but were unchanged or decreased 3 hours after a single session of neuromuscular electrical stimulation (Figure 2.4E,G, and H). ACADL was decreased after acute and chronic muscle training (0.94±0.031, p=0.098,

0.80±0.044, p=0.025, respectively), with a larger effect observed after chronic neuromuscular electrical stimulation training (Figure 2.4F).

*Tricarboxylic Acid Cycle, Oxidative Phosphorylation, and Mitochondrial Fission/Fusion*

The common substrate, acetyl CoA, following aerobic glycolysis or fatty acid oxidation is further oxidized within mitochondria via the tricarboxylic acid cycle and oxidative phosphorylation pathways. Genes responsible for monocarboxylic acid transport into the mitochondria (MPC1 (1.55±0.19, p=0.036) and MPC2 (1.55±0.17, p=0.034)) were increased after chronic neuromuscular electrical stimulation training and unchanged 3 hours after a single session of neuromuscular electrical stimulation (Figure 2.5A and B). A subset of rate limiting oxidative enzymes in the tricarboxylic acid (OGDH (1.50±0.092, p=0.007) and SDHB (1.54±0.081, p=0.004)) and oxidative phosphorylation (NDUFB1 (1.22±0.088, p=0.067), NDUFA2 (1.40±0.11, p=0.022), and CYC1 (1.34±0.13, p=0.066), COQ10A (1.49±0.14, p=0.024)) pathways were increased after chronic training but unchanged 3 hours after a single session of neuromuscular electrical stimulation (Figure 2.5C-H). MFF (1.35±0.14, p=0.062), OPA (1.67±0.27, p=0.074), MFN1 (1.36±0.25, p=0.22), and MFN2 (1.35±0.053, p=0.004) aid in regulating mitochondrial fission and fusion, and were increased with chronic training (Figure 2.5I-L).

*qPCR Validation*

We verified the expression levels of five genes (ABRA, EGR1, MYH7, NR4A3, and MSTN) using qPCR. Similar to the exon microarray results, ABRA, EGR1, and NR4A3 expression was increased following an acute dose of electrical stimulation but was decreased or unchanged in the chronically trained muscle (Figure 2.6A-C). Chronically training muscle resulted in a sustained increase of MYH7, while a single dose of activity minimally altered MYH7 expression (Figure 2.6D). Chronic and acute muscle stimulation

resulted in repression of MSTN, with a larger effect being seen within the chronically trained muscle (Figure 2.6E).

## Discussion

We examined the mRNA expression response after a single session of exercise with electrical stimulation in paralyzed soleus muscle and compared that to the mRNA expression profile of chronically trained paralyzed soleus muscle. The objective of this study was to advance our understanding of the influence of acute or chronic exercise on regulating gene expression important for metabolic health of skeletal muscle in humans with paralysis. The major findings of this study are 1) acute stimulation of paralyzed muscle regulates 117 biological pathways as compared to 35 in chronically trained muscle; 2) acute electrically induced exercise up regulates transcriptional, translational, and enzyme regulators of metabolic pathways that shift muscle toward an oxidative phenotype (PGC-1α, NR4A3, IFRD1, ABRA, PDK4); 3) long-term electrically induced exercise increased the chronic expression of glycolysis (PDHA1, PDHB, PDHX), fatty acid oxidation (ACADVL, ACAD8, ACAD9), tricarboxylic acid (MPC1, MPC2, OGDH, SDHB), and oxidative genes (NDUFB1, NDUFA2, CYC1, COQ10A); 4) long-term electrically induced exercise increased oxidative muscle fiber (MYH6, MYH7 MYL3, and MYL2) and mitochondrial fission/fusion (MFF, OA1, MFN1, MFN2) genes, but repressed glycolytic muscle (ACTN3, MYLK2, and MYL5) and muscle atrophy (MSTN) genes. Taken together, these findings support that the minimal dose (0.6 % of muscle activity per day) of electrically induced exercise used in this study was sufficient to initiate and chronically maintain several metabolic pathways in human paralyzed muscle. Whether preserving the metabolic capacity of skeletal muscle in humans after spinal cord injury improves systemic metabolic health remains unknown.

In 1995, we reported that the acutely paralyzed soleus muscle in humans is fatigue resistant and oxidative, but transforms into a highly fatigable, glycolytic muscle after 1 year of paralysis(Shields 1995). Subsequent long-term training intervention studies verified that there was a feasible dose of daily muscle exercise, induced by electrical stimulation, that could sustain bone mineral density of the underlying skeletal system (Dudley-Javoroski et al. 2012; Shields and Dudley-Javoroski 2006; Shields et al. 2006b) and phenotype of the soleus muscle including its size, fatigue resistance, muscle oxidative enzymes, reduced post activation potentiation, and reduced muscle contractile speeds (Adams et al. 2011; Shields et al. 2006a; Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006c). The high incidence of metabolic disease (diabetes) in individuals with spinal cord injury(Bauman et al. 1999a; Duckworth et al. 1980; Lavela et al. 2006), prompted us to question whether the transformation of paralyzed muscle to the glycolytic state contributes to the development of systemic metabolic syndromes consistent with skeletal muscle of people with diabetes(Stuart et al. 2013b). We discovered, in this study, that a moderate dose of acute and chronic electrical stimulation regulates common and distinct genes and pathways involved with glucose metabolism.

This study demonstrated that a single dose of electrically induced exercise stressed muscle and triggered the transcription of genes important for controlling metabolic pathways. Further, chronic training of paralyzed muscle resulted in a consistent increase of genes and proteins important for an oxidative, slow-twitch phenotype. It is noteworthy that skeletal muscle adaptation requires a repetitive and long-term (over a 1 year time) delivery of a stressor in order to up regulate the full complement of oxidative enzymes necessary to improve muscle endurance (Shields and Dudley-Javoroski 2006) and reduce muscle fat (Adams et al. 2011; Shields and Dudley-Javoroski 2007) (see Figure 2.1). The minimal dose needed to prevent skeletal muscle transformation after paralysis

is not known. The magnitude of the gene regulation after acute and chronic electrical stimulation in this study is striking considering the muscle was active for less than 0.6% of the day, while the remainder of the day the muscle experiences little contractile stress.

Two factors likely contributed to the magnitude of gene signaling based on the design of this protocol. First, the frequency of stimulation was set to create an unfused tetany, which generated ~ 50% of the maximal muscle force generating capacity, under isometric conditions. We chose this level of stimulation because we believed 50% of maximal force mechanically strains tissues that modulate muscle signaling and we wished to limit the total force of the muscle to a level that would not induce injury to the underlying skeletal and soft-tissues. Second, we supra maximally activated the muscle, which assured all fibers of the soleus muscle were activated. This level of muscle activity is unique and specific to electrical stimulation induced exercise. For example, this dose of exercise cannot be duplicated in humans under volitional control, as motor unit recruitment, via the size principle (Henneman 1985), and rate coding (Bigland-Ritchie et al. 1983) will not allow a volitionally driven muscle to be fully activated at a low firing frequency. Because electrical stimulation exercise recruits motor units differently from volitional exercise, it is risky to assume that training principles learned from normal volitionally driven exercise apply to paralyzed muscle exercised through electrical stimulation.

The unique method of stimulating paralyzed muscle induced the peroxisome-proliferator-activated-receptor-gamma-coactivator-alpha (PGC-1α) gene. Increased PGC-1α expression results in muscle hypertrophy, oxidative, and slow-twitch protein expression, while decreased levels lead to the conversion to glycolytic proteins and a transformation to a faster-twitch, less oxidative muscle fiber (Egan et al. 2010; Holmstrom et al. 2012;

Wallace et al. 2011). In able-bodied individuals, bouts of exercise have been shown to increase the expression of PGC-1α, with large increases seen when the muscle has been metabolically challenged (Subramanian et al. 2005). In this study, a single dose of electrical stimulation challenged the predominantly fast, glycolytic paralyzed muscle resulting in over a 5-fold increase in PGC-1α expression. Interestingly, the chronically trained soleus muscle demonstrated a sustained increase in PGC-1α expression when compared to the opposite untrained paralyzed muscle. However, the PGC-1α expression was far less than that induced just 3 hours after a single dose of electrical stimulation. The elevated PGC-1α expression in the chronically trained state suggests that muscle protein adaptations, induced by the repetitive challenge through chronic training, now prevents the buildup of metabolites (reactive oxygen species) that are known to initiate the cascade of events that follow PGC-1α transcription (Little et al. 2011b).

The nuclear orphan receptor-1 (also referred to as NR4A3) assists in regulating muscle metabolic phenotypes, with increased expression resulting in a more oxidative muscle fiber (Kawasaki et al. 2009; Pearen et al. 2012). Several environmental stressors have been shown to regulate NR4A3 expression including β-adrenoreceptors, cold, fatty acids, glucose, insulin, cholesterol, melanocortin, and thiazolidinediones (Kawasaki et al. 2009; Pearen et al. 2012). However, our single dose of exercise, induced by electrical stimulation, caused a >5 fold increase in NR4A3 mRNA 3 hours after exercise. Although, this study did not investigate the specific parameters of electrical stimulation that optimize the expression of NR4A3, we know that long-term use of this stimulation ultimately leads to less than a 1 fold increase in NR4A3 mRNA expression, suggesting that NR4A3 plays a role in initiating a metabolic shift, but may be less important during periods of inactivity.

Actin-binding Rho-activating protein (ABRA) has also been shown to be instrumental in triggering the transformation of muscle to a more oxidative muscle fiber phenotype through the activation of the PGC-1α pathway (Lamon et al. 2009; Wallace et al. 2011; Wallace et al. 2012). ABRA is located within the sarcomere and is thought to be a critical link in triggering cellular adaptions by transducing the mechanical stress to intracellular communication pathways (Arai et al. 2002; Wallace et al. 2012). Additionally, ABRA has been shown to translocate to the nucleus and may have act as a transcriptional co-activator or transcription factor (Arai et al. 2002; Wallace et al. 2011). Interestingly, ABRA expression has been show to decrease with immobilization and during disuse atrophy (Giger et al. 2009; Wallace et al. 2012) and seems to be increased in diabetic muscle (Jin et al. 2011; Wallace et al. 2012). However, the relationship between ABRA and insulin-sensitivity is not known.

We limited our analysis to genes with a large differential expression (>2 FC) compared to the untrained limb. However, limiting to a >2 FC eliminates transcripts that are differentially expressed but to a lesser magnitude. We correlated genes that did not meet the 2 FC thresholds but have important roles in regulating oxidative metabolism. MPC1 and MPC2 are two genes that control the transport of pyruvate from the cytosol to the mitochondria for aerobic oxidation (Bricker et al. 2012b). Interestingly, MPC1 and MPC2 were not altered 3 hours after exercise induced by electrical stimulation. However, with chronic training, both increased with a >1.5 FC compared to the untrained limb.

The mitochondrion is a critical organelle for skeletal muscle metabolism, generating over 90% of a cell's energy (Barbour and Turner 2014). The mitochondrial stress response contributes to the development of insulin resistance (Kim et al. 2008). In skeletal muscle, mitochondria form a complex network that may be an indicator of the metabolic health of

a muscle fiber. Mitochondria are regulated by the balance between mitochondrial fission and fusion(Barbour and Turner 2014). The lack of mitochondrial fusion and excessive mitochondrial fission contributes to mitochondrial dysfunction, decreasing the oxidative capacity of the muscle fiber (Barbour and Turner 2014). Chronic muscle training, using neuromuscular electrical stimulation, increased the expression of mitochondrial fusion genes. However, mitochondrial fission genes (DNM1L and INF2) (Iqbal et al. 2013) were unchanged (data not shown). Interestingly, the mitochondrial fission and fusion genes were minimally altered 3 hours after a single dose of muscle activity. However, a single dose of muscle activity significantly increased the expression of PGC-1α, which is a key initiator mitochondrial biogenesis. Taken together, repeated long-term exposure to the stress of muscle activity is important to cause cellular adaptations needed to alter the oxidative capacity of a muscle.

The findings support that exercise, through electrical stimulation, is a powerful method to stress skeletal muscle. Acutely, genes that function as transcriptional regulators are most heavily influenced. However after chronically training for over 1 year, there was a substantial increase in genes closely associated with an oxidative, slow-twitch phenotype. Exercised skeletal muscle has a high oxidative capacity, and can more rigorously respond to insulin and metabolize glucose (Kulkarni et al. 2012; Michael et al. 2001). The change in muscle insulin receptor sensitivity associated with routine exercise may, in part, explain several of the pathways regulated in this study (Babraj et al. 2009; Fritz et al. 2011; Little et al. 2011a; Little et al. 2011b; Winnick et al. 2008). For individuals with spinal cord injury, an ability to sustain the size and oxidative capacity of skeletal muscle may be paramount to improving overall metabolic health.

Future studies are underway to deliver interventions shortly after the injury that efficiently regulates skeletal muscle with the goal to optimize the health of people with paralysis. The optimal electrical stimulation parameters or dose (frequency, current intensity, duration, work-rest) to most efficiently elicit long-term cellular adaptations of paralyzed muscle are not known. Typically, the effectiveness of an electrical stimulation training program relies on long-term studies that culminate in the comparison of the muscles performance (fatigue, peak force, contractile properties, and potentiation) following several weeks of intervention. By targeting specific gene signaling pathways regulated by exercise, future studies may develop “scientifically grounded” electrical stimulation interventions before initiating expensive and long-term training programs to preserve or restore the metabolic capacity of paralyzed skeletal muscle. This study provides the first comparison of the mRNA transcripts that are influenced in paralyzed muscle after a single bout of electrical stimulation with that of a chronically trained human paralyzed muscle. Future studies examining various doses of electrical stimulation may selectively regulate quite different gene signaling pathways and be prescribed based on the needs of the individual with spinal cord injury (hypertrophy vs. metabolism).

*Methodological and Clinical Considerations*

The results of this study provide a “snapshot” into the effects that a defined dose of muscle activity has on gene regulation in human paralyzed muscle. We used an intervention to sufficiently “stress” the paralyzed muscle while also not injuring the underlying muscle or skeletal structures. In addition, we used a biopsy technique that minimized the amount of tissue harvested in order to reduce risk of secondary complications associated with venous stasis in humans with paralysis. We required nearly all of the harvested tissues to sufficiently extract enough RNA to examine cell signaling pathways, which was the primary objective of this study. However, this

precluded us from carrying out comprehensive proteomic studies and microscopy studies. Indeed, we do not know how long gene expression was altered after 3 hours or the dose needed to induce an actual change in protein. We do know that chronic training of ~10 minutes of activity/day caused a significant change in the functioning phenotype (fatigue, torque, underlying bone tissue) and a stable up regulation of several metabolic signaling pathways. While the number of participants in this study was not large, each subject had a control limb serving as a within subject comparison, constituting the identical genotype to contrast the effects of the electrical stimulation intervention. In addition, the long-term trained subjects, while limited in number, represent a novel example of the capacity for muscle tissue to adapt when exposed to a regular (> 1 year) but a modest dose of electrically induced exercise (0.6%/day).

*Conclusions*

A modest dose of acute electrical stimulation of paralyzed muscle regulated 117 biological pathways. The long-term effect of regular exposure to this same stress caused a stable up regulation of over 35 biological pathways. Acute activation of paralyzed muscle regulated several individual genes critical to initiating long-term change in muscle metabolism through transcription, translation, and enzyme regulation. The chronic gene pathways regulated were consistent with adaptations leading to oxidative fiber proteins and the development of slow twitch muscle speed, calcium handling, and hypertrophy. Both chronic training and acute muscle stimulation regulates cellular metabolism and cellular maintenance pathways, but cellular proliferation pathways are primarily regulated by repetitive chronic training. Taken together, these findings support that a minimal dose of electrical stimulation intervention (~10 minutes/day) delivered one time, or with long-term chronic use, regulates metabolic gene signaling pathways in humans with paralysis. We propose that efficiently improving the metabolic health of

people with paralysis will involve prescribing specific doses of electrically based exercise designed to target specific molecular pathways. Future studies are needed to determine if these training regimens attenuate the prevalence of diabetes in people with spinal cord injury.

## Tables

**Table 2.1 Top 10 expressed mRNA gene transcripts following an acute dose of neuromuscular electrical stimulation**

| Gene Symbol | Protein | Relative mRNA Level[a] |
|---|---|---|
| NR4A3 | nuclear receptor subfamily 4 group A member 3 | 12.45 ± 2.36 |
| EGR1 | early growth response protein 1 | 8.37 ± 1.48 |
| FOS | proto-oncogene c-Fos | 7.98 ± 3.43 |
| GEM | GTP-binding protein GEM | 6.59 ± 1.17 |
| KBTBD5 | kelch-like protein 40 | 6.25 ± 0.64 |
| ABRA | actin-binding Rho-activating protein | 5.98 ± 0.4 |
| IFRD1 | interferon-related developmental regulator 1 | 5.71 ± 0.73 |
| CYR61 | IGF-binding protein 10 | 5.70 ± 1.46 |
| PPARGC1A | peroxisome proliferator-activated receptor gamma coactivator 1-alpha | 5.46 ± 0.64 |
| MYC | myc proto-oncogene protein | 5.38 ± 1.02 |

[a]mRNA expression levels reported as group mean ± standard deviation of fold-change relative to the control limb

**Table 2.2 Top 10 expressed mRNA gene transcripts following chronic training of neuromuscular electrical stimulation**

| Gene Symbol | Protein | Relative mRNA Level[a] |
|---|---|---|
| MYH7 | myosin heavy chain 7 (slow isoform) | 11.69 ± 4.93 |
| MYL3 | myosin light chain 3 (slow isoform) | 9.07 ± 3.75 |
| PRUNE2 | protein prune homolog 2 | 7.01 ± 1.72 |
| MYH6 | myosin heavy chain 6 (cardiac muscle isoform) | 6.76 ± 2.5 |
| PMS2CL | PMS2-C terminal-like protein | 4.58 ± 0.96 |
| RSPO3 | R-spondin-3 | 4.49 ± 1.5 |
| AGBL1 | cytosolic carboxypeptidase 4 | 3.73 ± 0.85 |
| ENPP5 | ectonucleotide pyrophosphatase/phosphodiesterase family member 5 | 3.33 ± 0.36 |
| MRAP2 | melanocortin-2 receptor accessory protein 2 | 2.96 ± 0.3 |
| NPY6R | NPY6R neuropeptide Y receptor Y6 | 2.72 ± 0.68 |

[a]mRNA expression levels reported as group mean ± standard deviation of fold-change relative to the control limb

**Table 2.3 Top 10 repressed mRNA gene transcripts following an acute dose of neuromuscular electrical stimulation**

| Gene Symbol | Protein | Relative mRNA Level[a] |
|---|---|---|
| MSTN | growth/differentiation factor 8 | 0.56 ± 0.06 |
| ZNF30 | zinc finger protein 30 | 0.61 ± 0.04 |
| FAM217B | protein FAM217B | 0.62 ± 0.08 |
| YPEL2 | protein yippee-like 2 | 0.62 ± 0.03 |
| ZNF429 | zinc finger protein 429 | 0.63 ± 0.07 |
| POPDC2 | popeye domain-containing protein 2 | 0.63 ± 0.02 |
| RAB30 | ras-related protein Rab-30 | 0.63 ± 0.05 |
| TMEM242 | transmembrane protein 242 | 0.64 ± 0.16 |
| RBM43 | RNA-binding protein 43 | 0.64 ± 0.07 |
| ZNF92 | zinc finger protein 92 | 0.64 ± 0.06 |

[a]mRNA expression levels reported as group mean ± standard deviation of fold-change relative to the control limb

**Table 2.4 Top 10 repressed mRNA gene transcripts following chronic training of neuromuscular electrical stimulation**

| Gene Symbol | Protein | Relative mRNA Level[a] |
|---|---|---|
| ACTN3 | alpha-actinin-3 | 0.12 ± 0.03 |
| PVALB | parvalbumin alpha | 0.26 ± 0.19 |
| MSTN | growth/differentiation factor 8 | 0.33 ± 0.03 |
| SH3RF2 | putative E3 ubiquitin-protein ligase SH3RF2 | 0.36 ± 0.09 |
| HCN1 | potassium/sodium hyperpolarization-activated gated channel 1 | 0.36 ± 0.03 |
| AQP4 | aquaporin-4 | 0.37 ± 0.04 |
| SH2D1B | SH2 domain-containing protein 1B | 0.39 ± 0.09 |
| MYLK2 | myosin light chain kinase 2 (skeletal/cardiac muscle) | 0.4 ± 0.1 |
| MYL5 | myosin light chain 5 | 0.4 ± 0.07 |
| SLC22A3 | solute carrier family 22 member 3 | 0.42 0.03 |

[a]mRNA expression levels reported as group mean ± standard deviation of fold-change relative to the control limb

## Figures

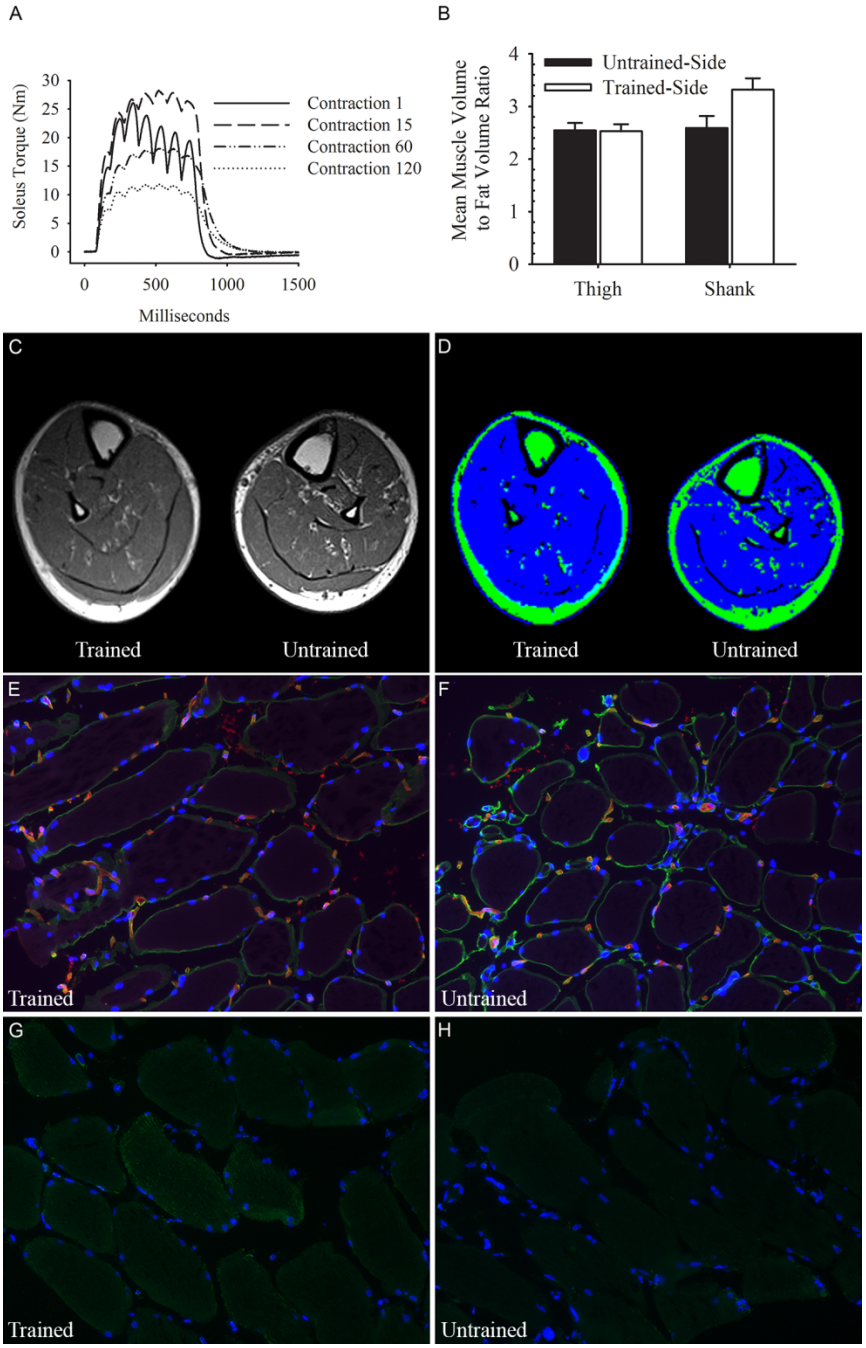


**Figure 2.1 Representative example for trained and untrained human paralyzed muscle**
(A) A representative example of the torque produced during the stimulation of a chronically paralyzed human soleus muscle, contractions 1, 15, 60, and 120 during the first bout of electrical stimulation are illustrated. (B) The ratio of muscle to adipose tissue from several MR images slices of the proximal shank and distal thigh after >7 years of unilateral soleus electrical stimulation training in subject 1. A representative MR Image slice of the trained and untrained lower leg before (C) and after (D) implementing the muscle and fat tissue segmentation algorithm. Immunofluorescence stain for collagen IV (green) in a chronically trained (E) and untrained (F) paralyzed muscle. Note the loss of collagen IV (green) in the chronically trained muscle. Immunofluorescence stain (green) for mitochondrial distribution in a trained (G) and untrained (H) paralyzed muscle.

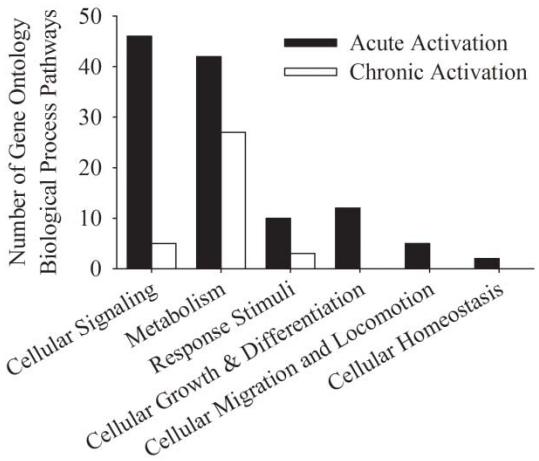


**Figure 2.2 GO biological process pathways upregulated by GSEA compared to non-stimulated limb**
117 pathways, primarily consisting of intra- and inter- cellular signaling pathways were upregulated 3-hours after a single session of exercise using neuromuscular electrical stimulation (Acute Soleus Stimulation). 35 pathways, primarily consisting of metabolic pathways, were upregulated in subjects that trained >7 years using neuromuscular electrical stimulation (Chronic Soleus Stimulation).

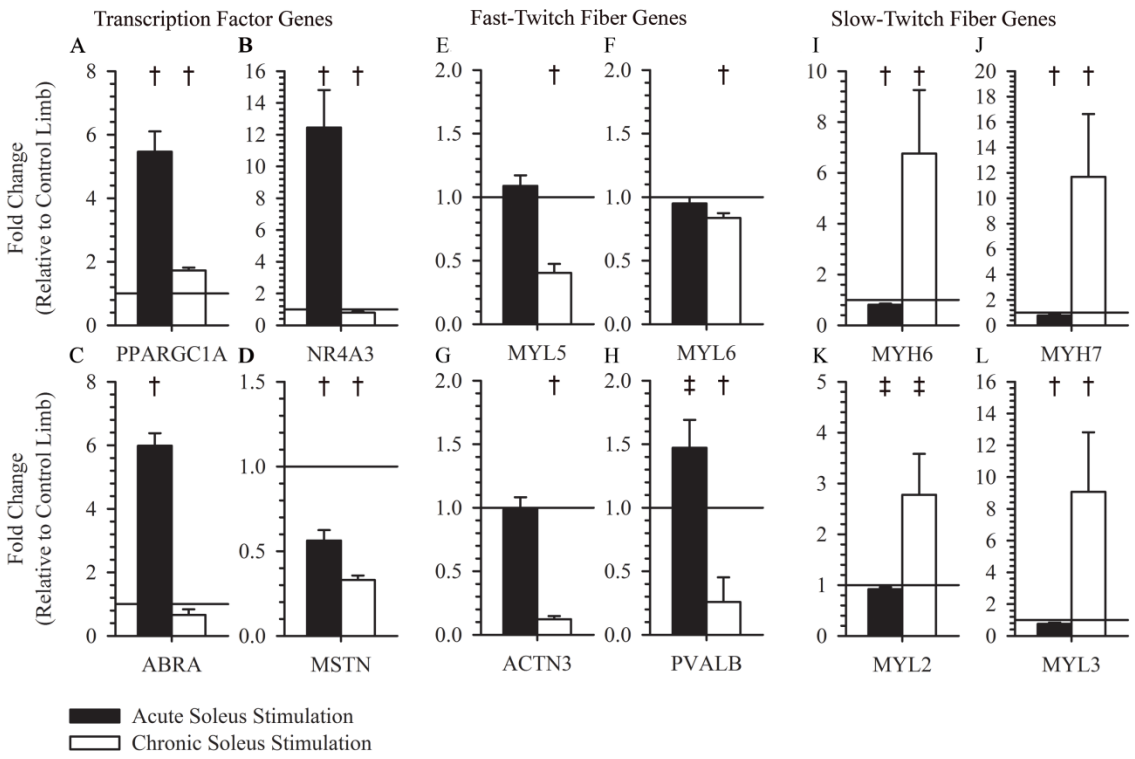


**Figure 2.3 Expression of transcription factor, fast-twitch fiber, and slow-twitch fiber genes following acute or chronic stimulation**

PGC-1α was increased 3 hours after a dose of muscle stimulation (5.46±0.64, p<0.001) and after >1 year of muscle training (1.73± 0.09, p<0.002) (A). NR4A3 was increased 3 hours after a dose of muscle stimulation (12.45±2.36, p<0.001), while it was decreased after >1 year of muscle training (0.79±0.06, p=0.046) (B). ABRA was increased after a single dose of muscle stimulation (5.98±0.40, p<0.001), but was unchanged after >1 year of soleus training (0.66±0.18, p<0.16) (C). MSTN was decreased 3 hours after a dose of muscle stimulation (0.56±0.06, p=0.002) and after >1year of muscle training (0.33± 0.03, p<0.001) (D). MYL5 (0.040±0.07, p=0.013), MYL6 (0.84±0.038, p=0.030), and ACTN3 (0.12±0.025, p=0.003) were all decreased after >1 year of muscle training (E, F, and G). There was no difference detected 3 hours after a dose of muscle stimulation for MYL5 (1.09±0.083, p=0.45). MYL6 (0.95±0.048, p=0.32), and ACTN3 (0.99±0.095, p=0.72) (E, F, and G). PVALB was increased after a single dose of muscle stimulation (1.47±0.22, p=0.074), but was decreased after >1 year of muscle training (0.26± 0.19, p=0.047) (H). MYH6 (6.76±2.50, p=0.030), MYH7 (11.69±4.93, p=0.025), MYL2 (2.78±0.80, p=0.063), and MYL3 (9.07±3.75, p=0.046) were increased after >1 year of muscle training, while they were decreased 3 hours after single session of muscle stimulation (0.81±0.04, p=0.0073, 0.77±0.073, p=0.030, 0.92±0.036, p=0.066, 0.76±0.078, p=0.037; respectively) (I, J, K, and L). † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

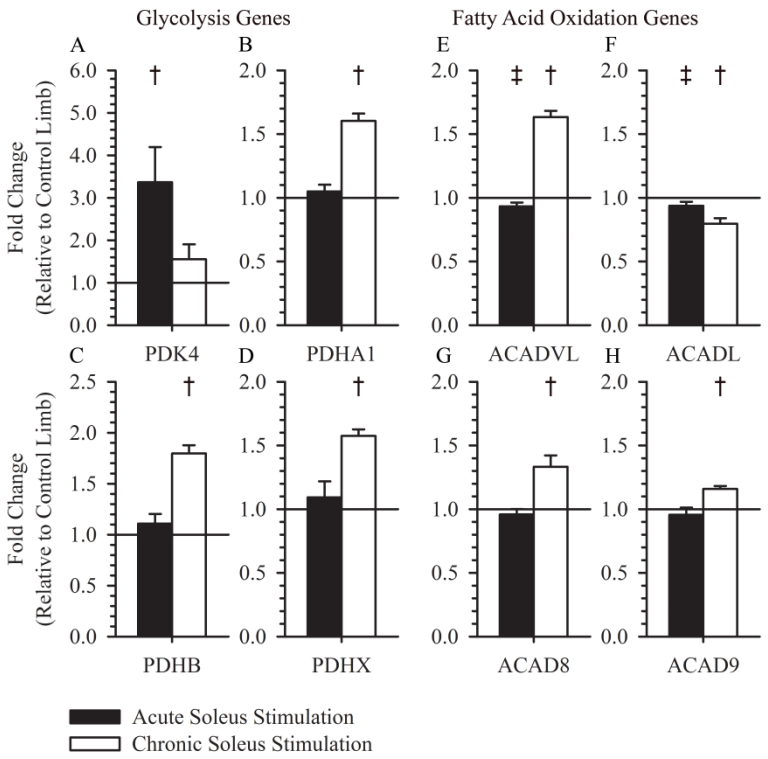


**Figure 2.4 Expression of glycolysis and fatty acid oxidation genes following acute or chronic stimulation**

PDK4 was increased 3 hours after a single session of muscle stimulation (3.37±0.83, p=0.008), but was unchanged after >1 year of soleus training (1.55±0.35, p=0.21) (A). PDHA1 (1.60±0.057, p<0.001) PDHB (1.80±0.08, p<0.001), and PDHX (1.57±0.05, p<0.001) were increased after >1 year of muscle training, but were unchanged 3 hours after a single session of muscle stimulation (1.05±0.05, p=0.46, 1.11±0.09, p=0.35, 1.09±0.13, p=0.59; respectively) (B, C, and D). ACADVL (0.93±0.03, p=0.064) was decreased 3 hours after a single session of muscle stimulation, but was increased after >1 year of muscle training (1.63±0.049, p=0.049) (E). ACADL (0.94±0.031, p=0.098) was decreased 3 hours after a single session of muscle stimulation and after >1 year of muscle training (0.80 and ACADL (0.94±0.031, p=0.098) were decreased 3 hours after a single session of muscle stimulation 0.044, p=0.025) (F). ACAD8 (1.33±0.089, p=0.023) and ACAD9 (1.16±0.023, p=0.006) were increased after >1 year of muscle training, but were unchanged 3 hours after a single dose of muscle stimulation (0.96±0.042, p=0.33, 0.96±0.06, p=0.39; respectively) (G and H). † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

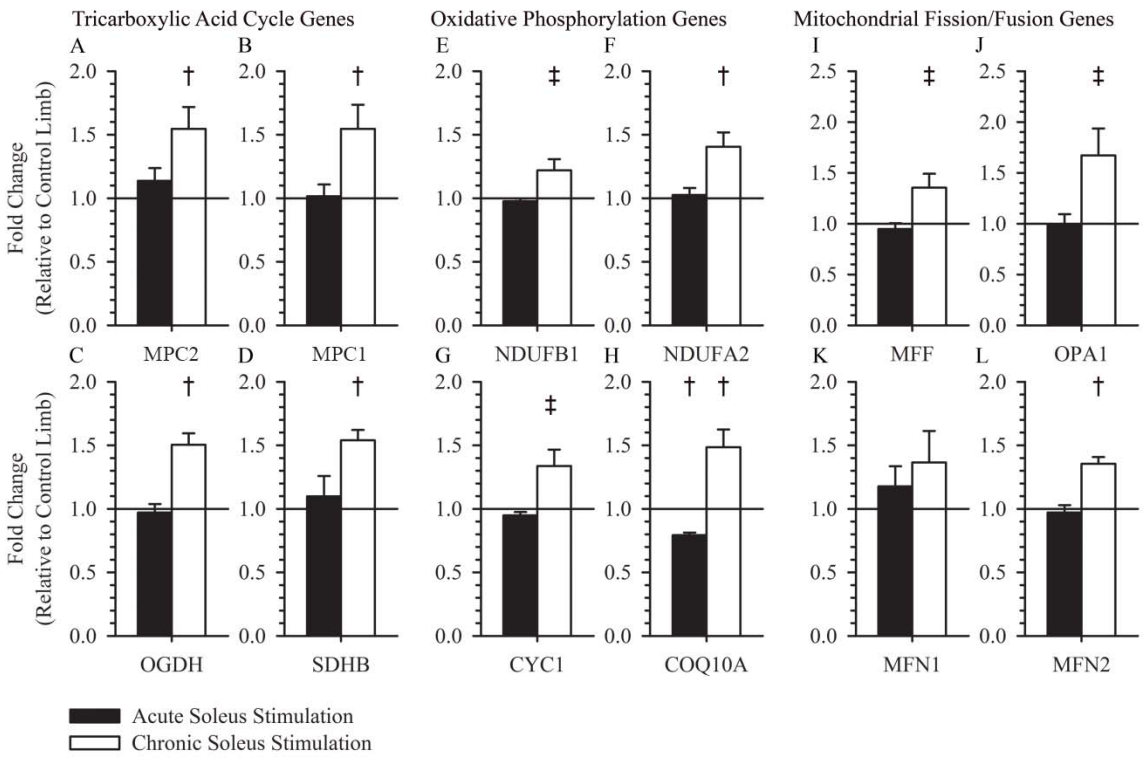


**Figure 2.5 Expression of tricarboxylic acid cycle, oxidative phosphorylation, and mitochondrial fission/fusion genes following acute or chronic stimulation**
MPC2 (1.55±0.19, p=0.036), MPC1 (1.55±0.17, p=0.034), OGDH (1.50±0.092, p=0.007), and SDHB (1.54±0.081, p=0.004) were increased after >1 year of muscle training, but were unchanged 3 hours after a single dose of muscle stimulation (1.14±0.099, p=0.25, 1.02±0.093, p=0.97, 0.97±0.067, p=0.58, 1.10±0.16, p=0.74; respectively) (A, B, C, and D). NDUFB1 (1.22±0.088, p=0.067), NDUFA2 (1.40±0.11, p=0.022), and CYC1 (1.34±0.13, p=0.066) were increased after >1 year of muscle training, but were unchanged 3 hours after a single dose of muscle stimulation (0.98±0.02, p=0.28, 1.03±0.05, p=0.72, 0.95±0.03, p=0.10; respectively) (E, F, and G). COQ10A was increased after >1 year of muscle training (1.49±0.14, p=0.024), but was decreased 3 hours after a single dose of muscle stimulation (0.79±0.021, p<0.001) (H). MFF (1.35±0.14, p=0.062), OPA (1.67±0.27, p=0.074), and MFN2 (1.35±0.053, p=0.004) were increased after >1 year of muscle training, but were unchanged 3 hours after a single dose of muscle stimulation (0.95±0.31, p=0.31, 1.00±0.78, p=0.77, 0.97±0.54, p=0.54); respectively) (I, J, and L). MFN1 was unchanged after >1 year of muscle training (1.36±0.25, p=0.22) and 3 hours after a single dose of muscle stimulation (1.18± 0.16, p=0.42) (K). † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

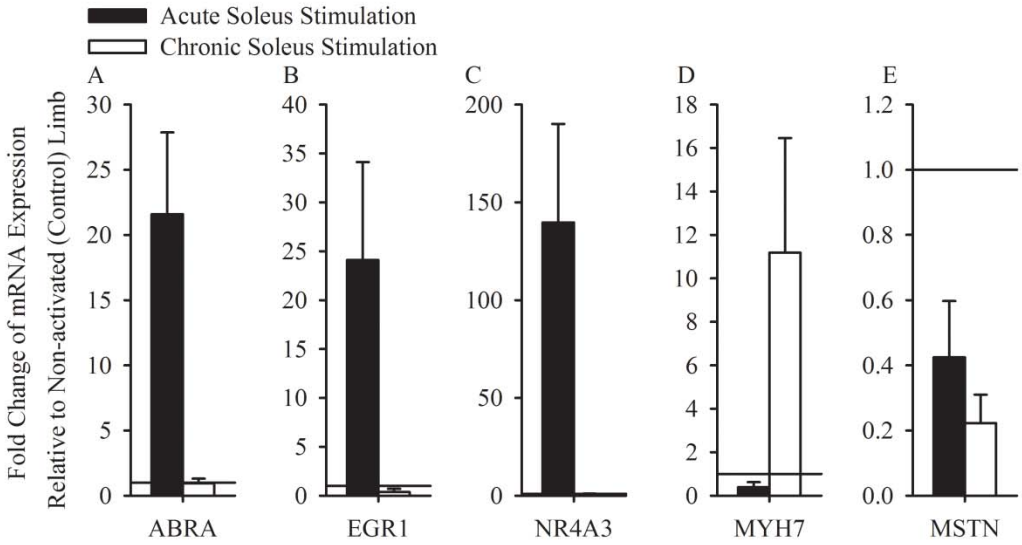


**Figure 2.6 RT-qPCR for a subset of genes in metabolic and hypertrophy pathways**
RT-qPCR analysis of mean ABRA (A), EGR1 (B), NR4A3 (C), MYH7 (D), and MSTN (E) levels for acutely stimulated muscles (black) and chronically trained muscles (white). (A-E) Data represented as mean ± standard error.

# CHAPTER 3 LOW-FORCE CONTRACTIONS INDUCE FATIGUE CONSISTENT WITH MUSCLE MRNA EXPRESSION IN PEOPLE WITH SPINAL CORD INJURY

## Introduction

After spinal cord injury (spinal cord injury) the muscular system atrophies and the fibers lose oxidative capacity (Grimby et al. 1976; Shields 1995; Shields and Chang 1997; Shields et al. 1997). The skeletal system becomes osteoporotic so that fractures may occur even during simple passive functional tasks (Fattal et al. 2011). There is over 50% loss of muscle mass after spinal cord injury, which removes important stress to bone (Eser et al. 2004), but also compromises metabolism as approximately 75% of glucose uptake occurs in skeletal muscle (Bjornholm and Zierath 2005). Healthcare workers are challenged to safely and efficiently promote exercise/activity for people with chronic spinal cord injury who are known to have secondary musculoskeletal and metabolic instability (Dudley-Javoroski and Shields 2006; LaVela et al. 2012).

Neuromuscular electrical stimulation (NMES) is one method used by clinicians to increase muscle activity in people with paralysis. The parameters selected during electrical stimulation are the current intensity, frequency, and pulse duration, which determine the force produced by skeletal muscle. When NMES is used to activate muscle of people with acute spinal cord injury (weeks to months after spinal cord injury), before musculoskeletal deterioration (osteoporosis), high intensity and high frequency stimulation is well tolerated (Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006c; Shields et al. 1997). However, 1-2 years after spinal cord injury, the

musculoskeletal system is compromised (Dudley-Javoroski and Shields 2008b; Shields and Dudley-Javoroski 2006) and there may be risk of injury when stimulating paralyzed muscle with high-forces (Hartkopp et al. 1998). Skeletal fractures currently are a leading public health risk to people with spinal cord injury (Heiden 2013; Krause et al. 2008). Importantly, people with paralysis may have better adherence to regular exercise if it can be delivered anytime of the day from the wheelchair.

An effective strategy, from a feasibility and safety perspective, for people with chronic spinal cord injury, would be to offer a low-force protocol that also challenges the paralyzed muscle as evident by muscle fatigue. If a low-force strategy is accomplished by using lower stimulation intensities, then a portion of the muscle is never recruited, and, therefore, not adapted with training. Conversely, by using a high stimulation intensity strategy (> 100-250 milliamps) with a low frequency (1-5 Hz), all muscle fibers can be recruited but at a single twitch force level. Most studies use stimulation frequencies that range from 15 to 50 Hertz (Burke et al. 1973; Shields 1995; Shields et al. 2006c; Shields et al. 1997; Stein et al. 1992), including one study that reported a fracture (Hartkopp et al. 1998). We located three studies that used a low-force stimulation protocol (< 5 Hz) for muscle training; however two of these studies were in animal models (Eisenberg and Gilai 1979; Metzger and Fitts 1987) and the only human case was on a single subject (Ryan et al. 2013).

The physiologic phenotype of skeletal muscle is commonly measured by the ability for a muscle to sustain a given force. Muscle fatigue, defined as the change in muscle force during repetitive activation, provides an index of the muscle's physiologic capacity. Muscles that have a mid- to high-fatigue resistance are considered oxidative and capable of using carbohydrates and lipids for fuel during long duration activity (Callister

et al. 2004; Martin et al. 1988); however, muscles that have a low fatigue resistance are glycolytic, primarily using anaerobic pathways and stored glycogen as fuel during short duration activity (Callister et al. 2004; Martin et al. 1988). Recently, fast, fatigable, glycolytic muscle has been linked to insulin receptor resistance and diabetes (Mootha et al. 2003; Palsgaard et al. 2009). However, there is a gap in the literature regarding the extent to which repetitive low-force contractions can challenge the chronically fast fatigable paralyzed muscle by inducing fatigue.

Repetitive stimulation of paralyzed muscle with higher force trains (15-50 Hz) causes extensive long duration muscle fatigue (Iguchi et al. 2008; Shields and Chang 1997; Shields et al. 1997). The long duration fatigue (also called low frequency fatigue) is thought to be due to impaired $Ca^{2+}$ release from the sarcoplasmic reticulum (Westerblad et al. 1993). A competing process is potentiation, whereby stimulating a muscle that was previously fatigued causes a “staircase phenomenon” or an incremental increase in force with continued activation (Rassier 2000; Rassier and Macintosh 2000). The mechanism for potentiation is believed to be due to increased sensitivity of $Ca^{2+}$ to the actin-myosin complex during subsequent twitches (Moore and Stull 1984). Several genes have been identified that play a prominent role in encoding proteins that control physiological responses during repetitive electrical stimulation. Specifically, mRNAs from genes for atrophy (MSTN) (Jones et al. 2004), fast-twitch, fatigable muscle (ANKRD1, MYH8 and MYCBP2) (Nakamura et al. 2002; Qin et al. 1994; Stevenson et al. 2006), slow twitch contractile properties (MYL3) (Alapat et al. 2009; Meyer et al. 2013), mitochondrial oxidative metabolism (SDHB, PDK2) (Jeong et al. 2012; Kita et al. 1990; Oh et al. 1996), and excitation-contraction coupling (RyR1) (MacIntosh et al. 2012) are known regulators of the muscle phenotype.

Our long-term goal is to determine if a safe and feasible low frequency/low-force muscle training protocol can induce long-term metabolic, physiologic, and molecular adaptations in humans with chronic spinal cord injury, ultimately translating into improved overall systemic metabolic health. In this study, we sought to test if muscle fatigue can be induced with a repetitive single twitch, low-force protocol and whether the physiologic phenotype is consistent with the underlying mRNA expression for select genes in people with and without chronic paralyzed muscle. We focus our molecular examination on select genes known to be associated with atrophy, fatigue, and fast glycolytic muscle phenotypes.

The purpose of this study was to compare the physiologic properties of chronically paralyzed and non-paralyzed muscle (fatigue and potentiation) during repetitive activation using a low-force generating stimulation protocol (3 Hz). Our secondary purpose was to examine if fatigability, induced via a low-force stimulation protocol, is consistent with the molecular expression of genes linked to each groups' muscle phenotype. We hypothesized that human chronically paralyzed muscle would be sensitive to a low frequency stimulation protocol consistent with the gene expression profile.

## Methods

*Subjects*

29 people, 9 men with a complete motor spinal cord injury (American Spinal Injury Association ASIA-A) and 20 people without spinal cord injury (10 men and 10 women) participated in a low frequency stimulation protocol (Table 3.1). The 9 subjects with spinal cord injury had increased tendon reflexes consistent with upper motor neuron

lesions. Some of the spinal cord injury subjects previously received neuromuscular electrical stimulation training; however, all spinal cord injury subjects had been discontinued any electrical stimulation for at least 1 year before participating in this study. Three subjects (subject 1, 3, and 6 with spinal cord injury) and 3 non-spinal cord injury subjects were biopsied. One subject (subject 6) underwent a biopsy 3 hours after a low-force protocol. This study was approved by the University of Iowa Human Subjects Office institutional review board. All subjects provided written informed consent before participation.

*Twitch Assessment of Fatigue*

Subjects sat in a wheel chair or standard chair with the testing limb ankle secured to a force transducer. The testing limb thigh and knee angle were set to 90°. The transducer apparatus consisted of a padded, semicircular metal plate that cupped the posterior surface of the leg. This plate was connected to a force transducer (1500ASK0299, Interface, Scottsdale, AZ) that was mounted to a rigid metal plate. The transducer could be adjusted vertically to suit the height of the subject's medial malleolus. A padded strap secured the tibia to the force transducer apparatus (Figure 3.1).

Self-adhesive 7cm x 13cm oval carbon electrodes (EMPI, Inc. St. Paul, MN) were adhered to the skin over the quadriceps muscles, consistent with a previously reported protocol (Dudley-Javoroski et al. 2008). In brief, the distal margin of the distal electrode was placed over the distal-most palpable border of the vastus lateralis. The proximal electrode was positioned as close to the inguinal crease as possible, with the medial-margin lateral to the adductor muscle group. Electrodes were connected via shielded cabling to a constant current electrical muscle stimulator unit (Digitimer model, DS7A, Digitimer, Welwyn Garden City, Hertfordshire, UK). Digital pulses controlled the

stimulator from a data-acquisition board (NI USB-6221BNC, National Instruments, Austin, TX) under software control (Labview 2012, National Instruments, Austin, TX). The stimulator was set to deliver 200μs pulses at 400 V, at intensities up to 300mA. All testing was conducted at intensities that elicited maximal twitch forces, verified by no increase in force with subsequent increase in stimulation intensity. Our non-spinal cord injury subjects were lean and achieved maximal contractions at around 100 mA. Because individuals with spinal cord injury typically have more resistance to stimulation (edema, subcutaneous fat), a higher current intensity was required to achieve the maximal contraction (~150 mA on average). The force and stimulator signals were amplified and sampled at a rate of 2,000 Hz using a customized software application.

*Experimental Protocol*

Single stimulus pulses were delivered at progressively increasing intensities up to 300 mA for the spinal cord injury and up to 100 mA for the non-spinal cord injury subjects to ensure supra maximal muscle stimulation. All stimulus pulses were widely spaced (10 s between pulse) to minimize muscle fatigue before beginning the testing procedure. The testing procedure consisted of 2 bouts. The first bout of the testing procedure consisted of a single train of 1000 stimulus pulses delivered at 3 Hz. The second bout consisted of a train of 200 stimulus pulses delivered at 3 Hz. The second bout followed a 5-minute recovery period. Our preliminary testing indicated 1000 pulses at 3Hz fatigued the muscle during bout 1. During bout 2, our preliminary studies indicated muscle potentiation was saturated by 200 pulses.

Muscle Physiological Property Analysis. We focused our analysis on two physiological variables consisting of the fatigue index (FI), and the potentiation index (PI). We also analyzed the change in the twitch speed properties by calculating a normalized twitch

rate of force development. The FI was defined as the ratio of the final peak force to the peak twitch force within a bout. The PI was defined as the ratio of the peak twitch force and the initial twitch force. To determine the FI and PI, the peak twitch force was defined as the maximum peak amplitude of the force signal for all twitches within a bout. The normalized rate of force development was determined by calculating the slope between 20% and 80% of the peak twitch force and then dividing the slope by the peak twitch.

*Muscle Biopsy, Exon microarray Procedure, Protein Analysis*

The biopsy procedure has been previously described (Adams et al. 2011). Briefly, a percutaneous muscle biopsy was taken from the vastus lateralis muscle using a Temno biopsy needle (T1420, Cardinal Health, Dublin, OH) under ultrasound guidance within a sterile field. Several passes of the needle were used to collect a wide sampling range within the muscle. Following harvest, muscle biopsy samples were immediately placed in RNALater (Ambion) for RNA extraction or flash frozen in liquid nitrogen for protein extraction and stored at -86°C until further use. RNA was extracted using the RNEasy Fibrous Tissue Kit (Qiagen). DNAse was included in the protocol to ensure absence of genomic DNA in final samples. RNA samples were eluted in water and quantified via a NanoDrop method. In addition, the quality of each sample was assayed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

Microarray hybridizations were performed at the University of Iowa DNA Facility. Briefly, 50 ng RNA was converted to SPIA amplified cDNA using the WT-Ovation Pico RNA Amplification System, v1 (NuGEN Technologies, San Carlos, CA, Cat. #3300) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN MinElute Reaction Cleanup column (QIAGEN Cat #28204) according to modifications from NuGEN. Four µg of SPIA amplified DNA were used to

generate ST-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies, Cat #2000) and again cleaned up with the Qiagen column as above. 5µg of this product were fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module, v2 (NuGEN Technologies, Cat. #4200) per the manufacturer's recommendations. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Human Exon 1.0 ST arrays (Part No. 900650), and incubated at 45º C for 18 h with 60 rpm rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), signal amplified with antistreptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade and data were collected using the GeneChip operating software (GCOS) v1.4.

The Affymetrix Human Exon 1.0 ST arrays were initially normalized and summarized with a Robust Multi-array Average (RMA) using Partek Genomic Suites (v6.6 © 2013 Partek, Inc., St. Louis, MO). Resulting exon expression intensities were further averaged to define the expression intensity for a specific gene. The spinal cord injury and non-spinal cord injury signal intensities, relative to the mean signal intensity, were compared to identify expression similarities and differences. Expression intensities were verified using several housekeeping genes (REEP5, PSMB2, C1orf43, and CMP25A), all resulting in similar gene intensity levels. We also calculated the fold changes for one subject who had the activated and the inactivated vastus lateralis biopsied 3 hours following a low-force protocol. The fold change was calculated as the ratio of the stimulated mRNA intensity/non-stimulated mRNA intensity from the same person.

We analyzed the protein levels for two genes differentially expressed between the spinal cord injury and non-spinal cord injury groups. Western blots were carried out to quantify protein concentrations for SERCA2 and SDHA. SERCA2 is associated with fatigue resistant skeletal muscle and SDHA is a subunit of a key enzyme in the citric acid cycle. The flash frozen biopsy samples were homogenized using a glass pestle in 1X PBS + 0.5% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche). Samples were vortexed thoroughly in the presence of detergent and agitated for two hours at 4°C. Finally, samples were centrifuged twice at 16,300xg for 10 minutes each to remove insoluble matter. Resulting supernatants were assayed using the Bradford method. Samples were loaded in equal quantities, separated via electrophoresis on ready gels (Bio-Rad), and blotted onto PVDF membranes. Membranes were blocked in 3% BSA in TBS + 0.1% Tween 20 (TBSTw) for one hour, then incubated at 4°C overnight in SDHA or SERCA2 (both from Cell Signaling, Danvers, MS), each diluted to 1:1,000 in block solution. Three rinses in TBSTw were performed before 1 hour incubation in HRP-conjugated anti-rabbit secondary antibody (GE Healthcare Life Sciences) diluted to 1:10,000 in block solution. Finally, membranes were rinsed three times in TBStw; protein standards and secondary antibodies were visualized using streptactin-HRP (Bio-Rad). Chemiluminescence results were developed using the ImmunStar WesternC Substrate (Bio-Rad) and results were quantified using the ChemiDoc XRS system and Quantity One software (Bio-Rad) and normalized to subject's GAPDH level.

*Statistical Analysis*

A two-way split plot ANOVA with repeated measures was used to compare the FI and PI between the spinal cord injury and non-spinal cord injury cohorts (Subject x Bout x FI or PI). We carried out *post hoc* testing using the Tukey testing procedure, which accounts for experimental type I errors for all pair wise comparisons. Spinal cord injury subjects

were tested bilaterally, providing a replicate sample that was included in the statistical model; however, the non-spinal cord injury subjects were unilaterally tested and did not have replicate samples. All statistical significance was unchanged, regardless of whether the replicate samples were included as independent samples. Gene and protein expression levels were tested using an independent t-test. A significance level of ($p<0.05$) was used for all testing procedures.

## Results

*Physiologic Response to the Low-force Protocol*

The max twitch force for people with spinal cord injury decreased from bout 1 to bout 2 by over 40% (46.55±27.88N to26.72±17.03N). Conversely, the max twitch force for people without a spinal cord injury decreased from bout 1 to bout 2 by only 15% (62.29±20.31N to 52.88±15.66). The paralyzed muscle was highly fatigable to the low-force stimulation protocol while the non-paralyzed muscle was not fatigable. The fatigue index (FI) was 0.21±0.27 and 0.91±0.01 for the spinal cord injury and non-spinal cord injury groups, respectively, at the end of bout 1 (Figure 3.2 A and B). After bout 2, the spinal cord injury group FI was 0.56±0.03 ($p<0.001$) and the non-spinal cord injury group remained at 0.97±0.007. The FI was ~80% and 50% lower for the spinal cord injury group when compared to the non-spinal cord injury group for bout 1 ($p<0.001$) and bout 2 ($p<0.001$), respectively (Figure 3.2 A and B). The normalized twitch rate of rise for the non-spinal cord injury group was unchanged from twitch 10 to twitch 1000 (12.8 $s^{-1}$ to 12.5 $s^{-1}$). Conversely, the normalized rate of rise was faster at the start (15.0 $s^{-1}$) but showed significant slowing to 11.1 $s^{-1}$ by the 1000$^{th}$ contraction for the spinal cord injury group. Because of the extensive fatigue, there were no measureable effects in bout 2 for the speed properties.

The chronic paralyzed muscle also showed a progressive increase in peak force during repetitive activation, 5 minutes after the muscle was fatigued. The potentiation index (PI) for the spinal cord injury group was unchanged in bout 1 (1.04 ±0.01), while the non-spinal cord injury cohort was increased to 1.36±0.04 in bout 1 (Figure 3.3A and B). However, in bout 2, the PI for spinal cord injury increased to 1.60±0.06 ($p<0.001$), while the non-spinal cord injury group decreased to 1.26±0.02 ($p=0.08$). The PI was significantly increased for the spinal cord injury group during bout 2 ($p<0.001$), when the muscle was in the fatigued state (Figure 3.3A and B).

*Atrophy and Fast Fatigable Gene Expression*

Both the FI and PI indicated that the low-force stimulation, induced by a low frequency stimulation protocol, differentiated the spinal cord injury and non-spinal cord injury phenotype. We next examined key mRNAs from genes known to regulate the skeletal muscle phenotype. We focused this analysis on four genes that are closely linked to muscle atrophy (MSTN) (Jones et al. 2004) and fast-twitch, glycolytic muscle properties (ANKRD1, MYH8 and MYCBP2) (Nakamura et al. 2002; Qin et al. 1994; Stevenson et al. 2006). The mRNAs for genes MSTN, ANKRD1, MYH8 and MYCBP2 were all up regulated in the spinal cord injury group ($p< 0.003$, 0.001, 0.026, and 0.001), respectively, consistent with an atrophied muscle made up of fast glycolytic fibers (Figure 3.4A). A plot of the FI, relative to the mRNAs expression levels, indicated that the low FI was consistent with the up regulation of these selected genes relative to the non-spinal cord injury group (Figure 3.4B).

*Oxidative Metabolism and E-C Coupling Gene Expression*

We examined four mRNAs for genes known to regulate slow contractile properties (MYL3) (Alapat et al. 2009; Meyer et al. 2013), oxidative metabolism (SDHB, PDK2) (Jeong et al. 2012; Kita et al. 1990), and excitation contraction coupling (RyR1) (MacIntosh et al. 2012). The mRNAs for genes MYL3, SDHB, PDK2, and RyR1 were all significantly repressed in the chronic spinal cord injury groups when compared to the non-spinal cord injury group ($p<0.001$, 0.002, 0.012, and 0.018), respectively (Figure 3.5A). These findings are consistent with muscle fibers that show rapid fatigue and post activation potentiation. A plot of the FI, relative to the gene expression levels, indicated that the low FI was consistent with the down regulation of these selected genes relative to the non-spinal cord injury group (Figure 3.5B).

*Muscle Protein Analysis*

We examined two proteins associated with muscle fatigue (oxidative metabolism) and excitation-contraction coupling calcium kinetics. Succinate dehydrogenase (SDH), a mitochondrial oxidative enzyme, was significantly reduced in the chronic spinal cord injury group ($p=0.021$, Figure 3.6A). Sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA2), a key protein facilitating calcium uptake during repetitive activation of paralyzed muscle, was also reduced in the spinal cord injury group ($p = 0.07$) (Figure 3.6B).

*Acute Gene Expression Response*

Three hours following the low-force protocol the mRNAs for MSTN, ANKRD1, MYH8 and MYCBP2 genes, associated with atrophy and glycolytic pathways, were repressed 150%, 140%, 130%, and 30%, respectively (Figure 3.7). The mRNAs for the MYL3

gene, associated with slow contractile properties was up regulated over 70%, while SDHB, PDK2, and RyR1 mRNAs were minimally altered 3 hours after the exercise.

## Discussion

The objective of this study was to determine if a low-force, repetitive activation protocol would induce fatigue and post fatigue potentiation. We discovered that 1000 stimulus pulses (twitches) delivered at a 3Hz stimulation frequency, caused fatigue of the paralyzed quadriceps muscle in people with long-term paralysis but did not fatigue the quadriceps of people without paralysis. The fatigability responses were consistent with each group's gene expression signatures. These findings support that a single twitch, low-force protocol, induced fatigue of the chronically paralyzed muscle while minimizing deleterious stress to the underlying skeletal system. Based on these findings, this protocol may offer a safe strategy to fatigue the chronically paralyzed muscle and regulate gene expression to enhance metabolic and physiological adaptations.

*Muscle Inactivity and Metabolic Health*

We previously described the physiological and histochemical properties of acute and chronic paralyzed muscle (Shields 1995; Shields and Dudley-Javoroski 2007; 2006), and developed a method to train acutely paralyzed muscle, to prevent bone loss in people with acute spinal cord injury (Dudley-Javoroski et al. 2012; Dudley-Javoroski and Shields 2013; Shields and Dudley-Javoroski 2007; 2006). However, individuals with chronic paralysis, who already have severe osteoporosis, may be limited in their capacity to train their paralyzed muscles because of fracture risk (Hartkopp et al. 1998). A protocol that minimizes force, but fatigues the muscle metabolic machinery, as examined in this study, may offer an alternative strategy to improve the health of chronically paralyzed muscle.

The link between muscle metabolic stability and fast glycolytic properties is now readily acknowledged. It has been shown that people with decreased insulin receptor sensitivity (diabetes) have a higher prominence of transformed fast glycolytic fatigable muscle fibers (Mootha et al. 2003; Palsgaard et al. 2009). The notion that skeletal muscle may be a key endocrine organ (Pedersen 2013) suggests that the systemic metabolic instability that is endemic to people with spinal cord injury (Lavela et al. 2006) is driven in part by the nearly 100% transformation of slow oxidative muscle fibers to fast fatigable muscle fibers (Shields 1995). A stimulation strategy to deliver repetitive activity to paralyzed muscle in an effort to down regulate glycolytic pathways and up regulate oxidative pathways, may improve muscle metabolism in people with spinal cord injury. In this study, we verified that a low-force protocol induced muscle fatigue and post fatigue potentiation. In addition, three hours after the low-force protocol, several of the gene expression profiles moved in a direction consistent with non-spinal cord injury muscle. Taken together, the delivery of this low-force protocol, on a regular basis, and perhaps throughout the day, may induce adaptations that move fast glycolytic spinal cord injury muscle towards that of non-spinal cord injury muscle.

New research in healthy people emphasize that sitting is an independent risk factor for metabolic disease (diabetes) (Chau et al. 2013; Healy et al. 2008). Episodic physical exercise in low-force people does not mitigate the effects of sitting (Chau et al. 2013; Healy et al. 2008). This finding underscores the inherent problem for people with spinal cord injury, who sit most of their life. Even one hour of physical activity per day did not compensate for the negative effects of excessive sitting on insulin sensitivity and plasma lipids in people without spinal cord injury (Duvivier et al. 2013). However, “minimal intensity” physical activity, distributed across the day for a longer duration, was most

effective at reducing insulin levels during glucose tolerance tests (Duvivier et al. 2013). The low-force generating 3 Hz protocol tested in this study, by virtue of the low risk to the skeletal system, may be tolerated several times a day to enhance glucose utilization in people with chronic paralysis and trigger a more stable systemic metabolic use of glucose. Importantly, this protocol may have high translatability because it of its ease of use at home and from the wheelchair. Recently, Carty et al. reported improvements in aerobic capacity and body composition using sub tetanic muscle contractions in humans with spinal cord injury (Carty et al. 2013; 2012), which supports the notion that low-force muscle exercise may induce systemic cardiovascular adaptations.

*High Stimulation Intensity with Low Frequency/Force Contractions*

Much of what we know about muscle adaptations originates from volitional studies (Rivera-Brown and Frontera 2012; Schoenfeld 2013). Because of the size principle, it is unnatural (or virtually impossible) to volitionally recruit the entire muscle at a low-force during isometric contractions (Henneman et al. 1965). In order to volitionally increase muscle force, we are required to recruit more motor units. The protocol used in this study recruited the entire muscle, but at a frequency that would not allow the twitches to be summated. This raises the question, what role does peak force play in fatigue and mechano-transduction in modulating gene expression? In this study, we observed an 88% (only 9% loss in non-spinal cord injury) loss of force after 1000 stimulus pulses delivered at 3Hz in chronic paralyzed muscle. Using a modified Burke Fatigue Protocol (20 Hz; 333 ms on; 667 ms off; 120 contractions), we previously induced an 83% loss of force in chronically paralyzed muscle, despite generating an initial force that was 300% higher than the force used in this study (Shields 1995). Accordingly, it appears that muscle fatigue, induced by electrical stimulation of paralyzed muscle, may be independent of the peak force. When we examine the number of pulses delivered in

several studies, we observe that most muscle fatigue occurs after approximately 500 stimulation pulses (Shields 1995). The principle that the number of stimulation pulses, and not force, determines the magnitude of fatigue in skeletal muscle was first proposed in 1983 (Marsden et al. 1983).

*Mechanisms Contributing to Fatigue*

The activation of a muscle fiber is a complex process and failure may occur at various sites during electrical stimulation. NMES triggers nerve activation that elicits acetylcholine (ACH) release to elicit an action potential over the sarcolemma, which propagates to the transverse tubules (T-tubules). The ryanodine (RyR) and dihydropyridine (DHPR) receptors sense the sarcolemmal action potential in the T-tubules and release calcium ($Ca^{2+}$) from the sarcoplasmic reticulum (SR). The $Ca^{2+}$ influx interacts with troponin, freeing actin to bind with myosin and generate force. Muscle relaxation requires sarcoplasmic $Ca^{2+}$ to be actively sequestered back in the SR against a diffusion gradient (active process). A calcium-activated adenosine triphosphatase enzyme (SERCA) actively pumps $Ca^{2+}$ into the SR, allowing troponin to inhibit actin-myosin interactions. Adenosine triphosphate (ATP), the energy currency in the cell, is phosphorylated to adenosine diphosphate (ADP) and ultimately adenosine monophosphate (AMP, and inorganic phosphorous ($P_i$). SERCA phosphorylates ATP to sequester $Ca^{2+}$ during muscle relaxation, while myosin ATPase regulates the rate that cross-bridges are cycled. A single stimulus pulse puts the muscle into an “active state” followed immediately by the “inactive state to return to the resting condition. When stimuli are repeated at a higher stimulation frequency (15-30 Hz), the sarcoplasmic $Ca^{2+}$ concentration remains elevated, causing the twitches to summate and reducing the need to sequester $Ca^{2+}$. Because muscle relaxation requires energy (active uptake of

calcium), the protocol in this study induced complete relaxation 1000 times, a factor that may be important in challenging the paralyzed muscle.

We previously demonstrated that changes in M-waves during fatigue of paralyzed muscle was independent of the change in muscle force (Chang and Shields 2002; Shields 1995; Shields et al. 1998; Shields et al. 1997). Hence, we do not believe that this single twitch protocol induced fatigue by compromising the neuromuscular transmission system. Thus, several underlying mechanisms including $Ca^{2+}$ transient dysregulation, myosin light chain phosphorylation, and the accumulation of metabolic products may all have contributed to the fatigue observed in this study (Duchateau et al. 1987; Eisenberg and Gilai 1979; Fauler et al. 2012; MacIntosh et al. 2012). It is likely that the repeated muscle twitches triggered the buildup of byproducts of ATP phosphorylation (AMP and $P_i$), which decreased the RyR sensitivity and the subsequent release of $Ca^{2+}$ (Duchateau et al. 1987; Eisenberg and Gilai 1979; Fauler et al. 2012; MacIntosh et al. 2012). Given the robust difference in the RyR1 gene expression between the spinal cord injury and non-spinal cord injury muscle, we reason that paralyzed muscle has a limited capacity to repetitively induce the active and resting states of the muscle during a single twitch. The capacity for substrates like glucose and fatty acids to be used as an energy source (oxidative pathways) to decrease the rate of byproduct (AMP and P) production is unknown, but clearly regulated by enzymes under molecular control (MYL3, SDHB, and PDK2). Consistent with our mRNA studies, these genes are significantly down regulated in the chronically paralyzed muscle. The mitochondria are responsible for aerobic metabolism and are the major contributor to ATP production in fatigue-resistant muscle (Jacobs et al. 2013). Inactive muscle adapts by decreasing muscle size and mitochondrial number (Stephenson and Hawley 2013; Zierath and Hawley 2004). We recently demonstrated, in individuals with acute spinal cord injury, that higher force

training protocols (15 Hz) up regulate several genes that promote mitochondrial enzymes and glucose utilization (Adams et al. 2011). Interestingly, three hours after the low-force stimulation protocol in this study, all four genes that regulate atrophy and glycolytic properties were down regulated, while one gene that regulates oxidative properties was up regulated. Although the long-term training studies using a low frequency protocol have not been undertaken, we hypothesize, based on the responses from this study, that long-term adaptations are possible. Importantly, these adaptations are detected early by this molecular analysis, and may be an important strategy to optimize the stimulation dose to enhance the muscle health of people with chronic spinal cord injury.

*Mechanisms Contributing to Post Fatigue Potentiation*

Muscle force output may be modulated by the interaction between potentiation and fatigue (Rassier 2000; Rassier and Macintosh 2000). Activating a rested muscle phosphorylates myosin regulatory light chains (RLC), increasing sensitivity to $Ca^{2+}$ (MacIntosh et al. 2012; Moore and Stull 1984; Tubman et al. 1996; Tubman et al. 1997; Vandenboom and Houston 1996). The increased sensitivity leads to more actin-myosin interactions and increase the total force output (MacIntosh et al. 2012; Moore and Stull 1984; Tubman et al. 1996; Tubman et al. 1997; Vandenboom and Houston 1996). Continual activation of the muscle phosphorylates RyR, decreasing $Ca^{2+}$ release, mitigating the increased force obtained from RLC phosphorylation. Our findings were consistent with the notion that long duration fatigue compromises the rate of phosphorylation of chronically paralyzed muscle.

*Summary and Conclusions*

This study demonstrated that a low-force single twitch stimulation protocol fatigued the human chronically paralyzed muscle. The fatigue index, the potentiation index, and speed properties responded in a manner consistent with the mRNAs expressed for several genes known to regulate the physiological response to repetitive stimulation. A single bout of this low-force protocol promoted the mRNAs of genes in a direction consistent with the non-spinal cord injury subjects. Because individuals with spinal cord injury develop 1) severe osteoporosis, 2) have limited muscle activity, 3) impaired muscle glucose utilization, and 4) diabetes, this low-force activation protocol may present a novel strategy to mitigate the incidence of metabolic disease in a convenient and safe manner. How training with a low-force protocol affects muscle is unknown. Future studies are necessary to determine the longitudinal value of regular low-force exercise in comparison to other methods to sustain the overall health of people with spinal cord injury.

## Table

**Table 3.1 Chapter 3 subject characteristics**

| Subject | Gender | Age | Injury Level | Years post-injury |
|---|---|---|---|---|
| SCI 1 | M | 49 | T4 | 5 |
| SCI 2 | M | 63 | T12 | 4 |
| SCI 3 | M | 19 | T8 | 3 |
| SCI 4 | M | 27 | T8 | 7 |
| SCI 5 | M | 45 | C5 | 26 |
| SCI 6 | M | 31 | T10 | 5 |
| SCI 7 | M | 22 | C4-6 | 1 |
| SCI 8 | M | 33 | T5 | 9 |
| SCI 9 | M | 35 | T9 | 13 |
| NON 1 | M | 24 | - | - |
| NON 2 | F | 24 | - | - |
| NON 3 | F | 24 | - | - |
| NON 4 | M | 26 | - | - |
| NON 5 | F | 24 | - | - |
| NON 6 | F | 24 | - | - |
| NON 7 | M | 24 | - | - |
| NON 8 | M | 24 | - | - |
| NON 9 | M | 25 | - | - |
| NON 10 | M | 25 | - | - |
| NON 11 | F | 23 | - | - |
| NON 12 | F | 23 | - | - |
| NON 13 | M | 25 | - | - |
| NON 14 | F | 23 | - | - |
| NON 15 | M | 24 | - | - |
| NON 16 | M | 25 | - | - |
| NON 17 | F | 22 | - | - |
| NON 18 | M | 23 | - | - |
| NON 19 | F | 24 | - | - |
| NON 20 | F | 24 | - | - |

Subject with and without spinal cord injury are denoted by SCI and NON, respectively.
M, men; F, women; C, cervical; T, thoracic.

## Figures

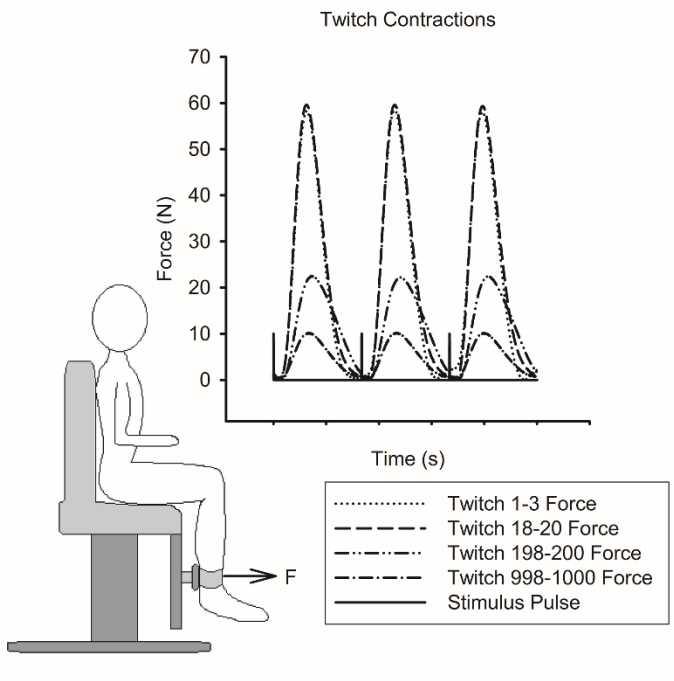


**Figure 3.1Schematic of measurement apparatus and representative example**
Torque measurement apparatus, with adjustable load cell and stabilization cuff (bottom). Representative twitches at the start, the peak, and the end of the first bout of the 3Hz stimulation protocol in paralyzed muscle (top). The initial and peak twitches show little difference in amplitude, but the later twitches show a decrease in amplitude.

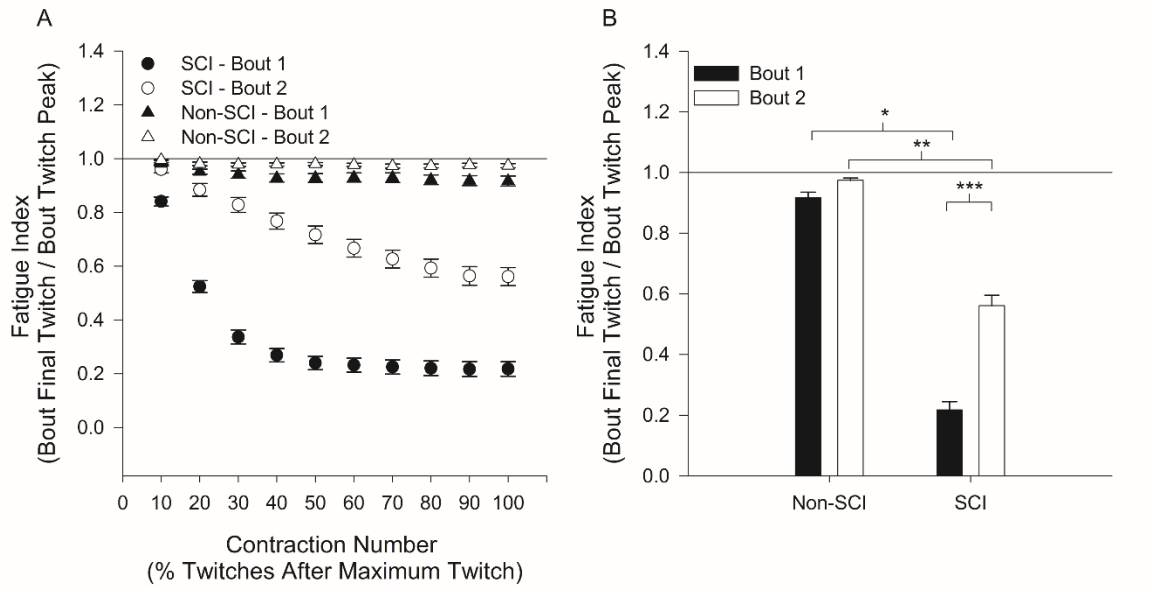


**Figure 3.2 Fatigue index assessment**
(A) The group means and standard errors for the fatigue index (FI), as a function of the maximum twitch, for bout 1 and bout 2. The twitches after the maximum twitch within each bout were grouped in bins of 10% of the remaining twitches. (B) The mean fatigue index (FI) for each group for bout 1 and bout 2. * significant difference between spinal cord injury and non-spinal cord injury for bout 1; ** significant difference between spinal cord injury and non-spinal cord injury for bout 2; *** significant difference between bout 1 and 2 for the spinal cord injury group.

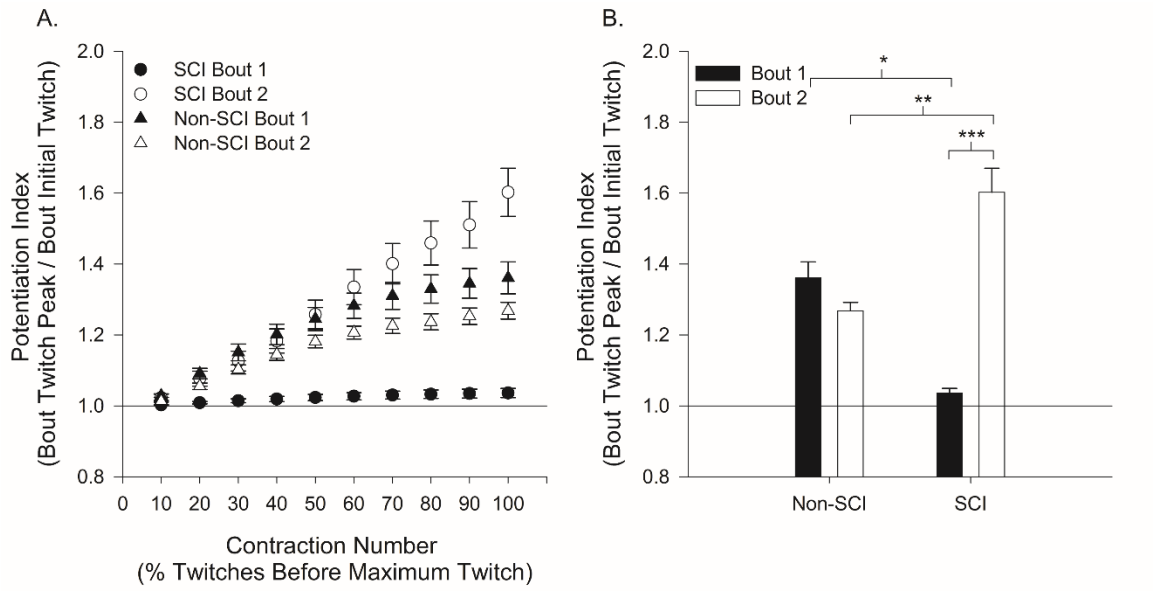


**Figure 3.3 Potentiation index assessment**
(A) The group means and standard errors for the potentiation index (PI), as a function of the maximum twitch, for bout 1 and bout 2. The twitches before the maximum twitch within each bout were grouped in bins of 10% of the preceding twitches. (B) The potentiation index (PI) for each group for bout 1 and 2. * significant difference between spinal cord injury and non-spinal cord injury for bout 1; ** significant difference between spinal cord injury and non-spinal cord injury for bout 2; *** significant difference between bout 1 and 2 for the spinal cord injury.

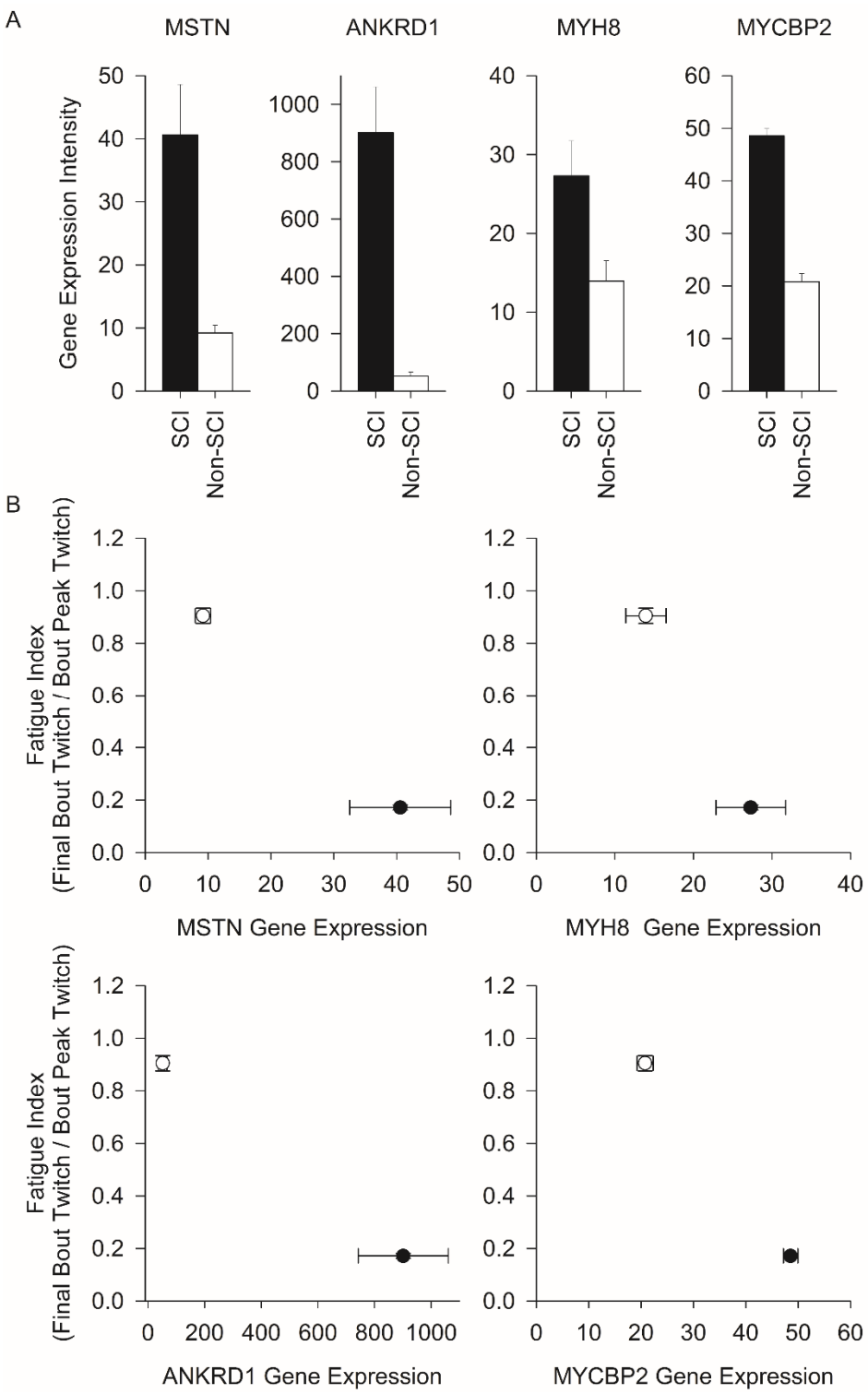


**Figure 3.4 Relationship between fatigue index and fast-twitch muscle genes**
(A) The relative gene expression intensity for MSTN, ANKRD1, MYH8, MYCBP2 between chronic spinal cord injury and non-spinal cord injury muscle phenotype. (B) A bidirectional standard error plot of the fatigue index versus the relative gene expression intensity (MSTN, ANKRD1, MYH8, and MYCBP2) for the chronic spinal cord injury phenotype (closed circle) and non-spinal cord injury phenotype (open circle). The relative expression intensity for all genes were significant at the $p<0.05$ level (see text for details).

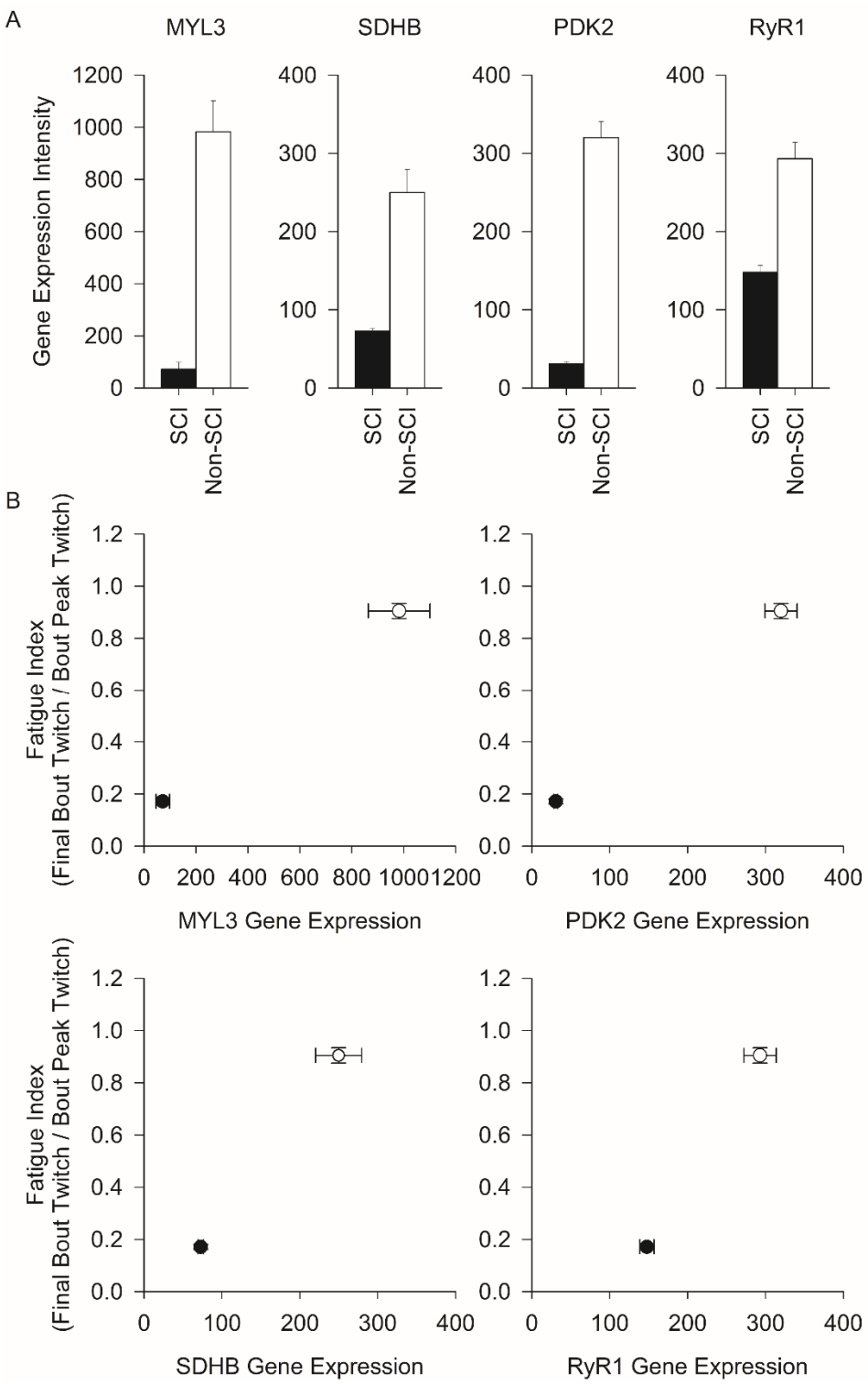


**Figure 3.5 Relationship between fatigue index and slow-twitch muscle genes**
(A) The relative gene expression intensity for MYL3, SDHB, PDK2, and RYR1 between a chronic spinal cord injury and non-spinal cord injury muscle phenotype. (B) A bidirectional standard error plot of the fatigue index versus the relative gene expression intensity (MYL3, SDHB, PDK2, and RYR1) for the chronic spinal cord injury phenotype (closed circle) and non-spinal cord injury phenotype (open circle). The relative expression intensity for all genes was significant at the $p<0.05$ level (see text for details).

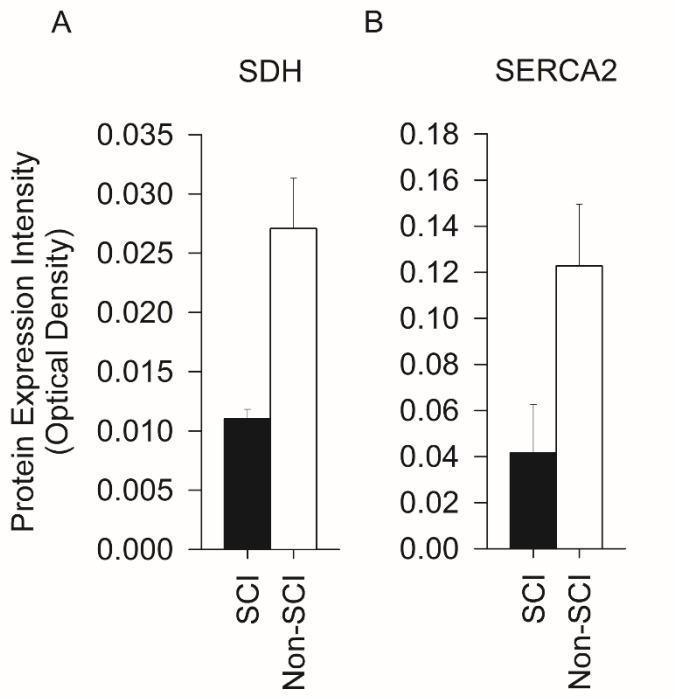


**Figure 3.6 Relationship between fatigue index and oxidative protein expression**
The relative protein expression level of SDH, an oxidative enzyme in the citric acid cycle which is partially translated by SDHB, was significantly depressed in chronic spinal cord injury compared to non-spinal cord injury at the $p<0.05$ level (A). The relative protein expression level of SERCA2, a calcium transport protein associated with slow-twitch muscle and the associated RyR1, was significantly depressed in chronic spinal cord injury compared to non-spinal cord injury at the $p<0.10$ level (B).

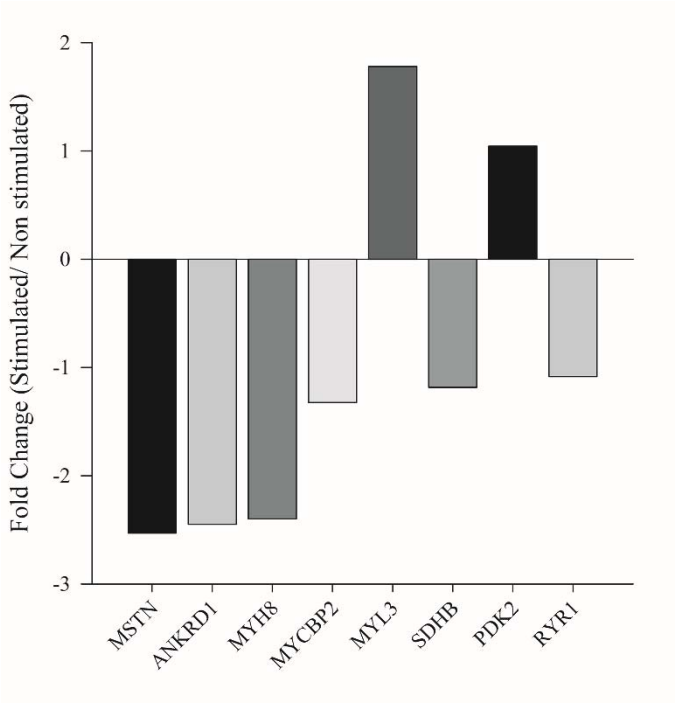


**Figure 3.7 Representative example of acute gene expression after single dose of low-frequency stimulation**
The gene expression changes from a single subject (subject 6) three hours after a low-force stimulation protocol. The expression of MSTN, ANKRD1, MYH8, MYCBP2, MYL3, SDHB, PDK2, and RYR1 is shown as the fold change difference between the stimulated muscle relative to the non-stimulated muscle.

# CHAPTER 4 ACUTE LOW-FORCE EXERCISE REGULATES METABOLIC GENE EXPRESSION IN PARALYZED MUSCLE

## Introduction

The musculoskeletal system enters a state of metabolic chaos after a spinal cord injury (Dudley-Javoroski and Shields 2008b). Skeletal muscle atrophies and transforms into a fast-twitch, glycolytic phenotype (Dudley-Javoroski and Shields 2008b; Shields 1995; Shields and Chang 1997; Shields and Dudley-Javoroski 2006; Shields et al. 1997) and the skeletal system deteriorates culminating in 2-4% loss of bone per month for first 12 months after the injury (Dudley-Javoroski et al. 2013; Dudley-Javoroski et al. 2012; Dudley-Javoroski and Shields 2013; 2008a). Systemic metabolic inflexibility, defined as a limited capacity to regulate glucose in the body, triggers a cascade of catabolic events that ultimately compromises the health of people with spinal cord injury (Bjornholm and Zierath 2005; Groah et al. 2011). We now understand that the transformation of skeletal muscle fibers from a slow oxidative to a fast glycolytic state yields a tissue that is metabolically less flexible (less sensitive to insulin) (Daugaard et al. 2000; Henriksen et al. 1990; Song et al. 1999; Stuart et al. 2013b), and may contribute to the prevalence of secondary complications experienced by people with spinal cord injury.

An inflexible muscular system is a primary characteristic of the diabetic phenotype (Bjornholm and Zierath 2005; Holmstrom et al. 2012; Jakobsen and Reske-Nielsen 1986; Stuart et al. 2013b) as up to 70% of glucose utilization may occur at the skeletal muscle level (Bjornholm and Zierath 2005). People with spinal cord injury have an impaired ability to activate their skeletal muscle voluntarily, which contributes to their

overall poor metabolic state. Confounding their condition is the severe osteoporosis, which affects the ability to apply high frequency evoked muscle forces via electrical stimulation.

Neuromuscular electrical stimulation is a time-honored method that challenges the physiological state of skeletal muscle tissue. Historically, a primary goal of electrical stimulation training is to increase the size (hypertrophy) of the underlying skeletal muscle (Dudley-Javoroski and Shields 2008b; Mahoney et al. 2005b; Shields and Dudley-Javoroski 2006). (Hartkopp et al. 1998; McHenry and Shields 2012)Recent emerging evidence for non-disabled people supports that long duration, low-force physical activity may have a greater stabilizing influence on a muscle's metabolic state as compared to short duration high intensity activity, even if the total energy expenditure is identical (Chau et al. 2013; Dunstan et al. 2012; Healy et al. 2008).

We sought to understand whether the same amount of electrical stimulation energy (number of pulses at the same intensity) across two different frequencies would yield different fatigue and regulation of metabolic genes in skeletal muscle. For example, at a low frequency of stimulation (5 Hz), paralyzed muscle is not fused so that the force is low; however, at a higher stimulation frequency (20 Hz), paralyzed muscle is fused, and a higher muscle force develops. We controlled the peak muscle force for each frequency by testing the muscle in a shortened position; assuring the safety of the underlying skeletal system. Because a higher stimulation frequency sustains the calcium transient as compared to the lower frequency stimulation, this study probed the question as to whether the frequency of calcium release (and subsequent muscle force) regulates key genes associated with muscle metabolism (Chin 2004; Marsden et al. 1983; Rose et al. 2006). To date, one study suggests that a high intensity muscle force is a pre-requisite

to modulate PGC-1α, a major transcription factor regulating muscle metabolism through mitochondria biogenesis (Egan et al. 2010).

Our goal is to examine key groups of genes known to be associated with the diabetic phenotype of skeletal muscle (Bjornholm and Zierath 2005; Mootha et al. 2003; Palsgaard et al. 2009). Therefore, we focused on acute stress genes associated with oxidative transcription (PGC-1α, NR4A3, IFRD1, ABRA) (Egan et al. 2010; Kawasaki et al. 2009; Mootha et al. 2003; Pruitt et al. Oct. 9, 2002). We next examined genes that are typically longer adapting that are known to regulate muscle contractile speeds (fast: MYL5, MYL6, ACTN3, PVALB, slow: MYH6, MYH7, MYL2, and MYL3) (Ogura et al. 2008; Pruitt et al. Oct. 9, 2002; Qin et al. 1994). Finally, we focused on genes that regulate metabolic pathways such as glycolysis (PDK4, PDHA1, PDHB, PDHX(Brown et al. 2004; Pruitt et al. Oct. 9, 2002), fatty acid oxidation (ACADVL, ACADL, ACAD8, ACAD9)(Pruitt et al. Oct. 9, 2002; Strauss et al. 1995), tricarboxylic acid cycle (MPC1, MPC2, OGDH, SDHB)(Bricker et al. 2012a; Pruitt et al. Oct. 9, 2002), oxidative phosphorylation (NDUFB1, NDUFA2, CYC1, COQ10A )(Gaignard et al. 2013; Pruitt et al. Oct. 9, 2002), and mitochondrial remodeling (MFF, OPA1, MFN1, and MFN2) (Brown et al. 2004; Iqbal et al. 2013; Palikaras and Tavernarakis 2014; Pruitt et al. Oct. 9, 2002).

Accordingly, the purpose of this study was to compare the effect of low and high frequency stimulation on muscle fatigue and metabolic gene expression in human paralyzed muscle. We expected that the low-force stimulation (5 Hz) would induce significant fatigue and up regulate key genes associated with metabolism. If supported this intervention offers a strategy to regulate skeletal muscle in people with severe osteoporosis from spinal cord injury.

## Methods

*Subjects*

Twelve subjects with chronic (> 1 year) and complete spinal cord injury (ASIA A) had the quadriceps twitch force assessed before and after electrical stimulation with a 5 Hz or 20 Hz protocol on two separate days (Table 4.1; Figure 4.1AB). A subset of 6 randomly chosen subjects participated in two additional sessions where a vastus lateralis muscle biopsy was performed bilaterally 3 hours after a unilateral session of 5 Hz or 20 Hz electrical stimulation; each session separated by > 1 month (Figure 4.1CD). During each session, only one leg was stimulated and the opposite leg served as a control. No subject reported previous participation in electrical stimulation for at least 6 months prior to this study. We excluded subjects with a history of lower limb fracture, peripheral nerve injury, pressure ulcers, or history of muscle injury. We obtained consent from all subjects as per the University of Iowa institutional review board approval.

*Twitch Force Assessment of Fatigue*

The muscle twitch force before and after each stimulation protocol (5 Hz or 20 Hz) was measured using a custom apparatus, as previously described (Petrie et al. 2014a). We used a lower stimulation frequency of 3 Hz to assess the single twitch properties before and after the 5 and 20 Hz protocols as depicted in Figure 4.1A and B. We chose the 3 Hz stimulation frequency because the muscle remains un-fused at this frequency even after fatigue has induced significant change in the muscle contractile speed (Petrie et al. 2014a). We obtained all 3 Hz generated muscle forces with the subject positioned in a chair, with the hip, knee, and ankle flexed to 90° with the ankle secured in series to a load cell (1500ASK0299, Interface, Scottsdale, AZ). Two 7cm x 13cm oval carbon electrode pads (EMPI, Inc. St. Paul, MN) were placed over the proximal and the distal

anterior thigh to deliver stimulus pulses to the quadriceps muscle, as previously described (Dudley-Javoroski et al. 2012; Dudley-Javoroski and Shields 2013; Petrie et al. 2014a). A constant current stimulator (Digitimer model, DS7A, Digitimer, Welwyn Garden City, Hertfordshire, UK) delivered 200μs pulses under software control. A data acquisition board (NI USB-6221-BNC, National Instruments, Austin, TX) digitized the analog signals from the load cell and stimulator at a sampling rate of 2000 Hz.

To warm up the muscle and establish the supra maximal stimulation intensity, we delivered single stimulus pulses at increasing intensities (maximal capacity: 300mA). When there was no further increase in muscle twitch force, with increased stimulation intensity, we increased the stimulation current an additional 25% to assure supra maximal muscle stimulation. We assumed that we activated nearly all muscle fibers as supported by our previous M-wave analysis (Adams et al. 2011; Coffey and Hawley 2007; Dudley-Javoroski and Shields 2008b; Shields 1995; 2002). Three minutes after we established supra maximal stimulation, we delivered a single bout of 200 stimulus pulses at a 3 Hz stimulation frequency (Petrie et al. 2014a). We delivered this protocol 5 minutes before and immediately after the 5 Hz or 20 Hz protocol on different days as shown in our protocol timeline (Figure 1AB). We analyzed the peak twitch force to determine the physiological influence of the 5 Hz and 20 Hz stimulation.

*Test Stimulation Protocols (5 Hz and 20 Hz)*

The knee was extended (10 degrees short of being straight) for the delivery of the 5 Hz and 20 Hz protocols. This position enabled us to minimize the overall peak force that develops at the 20 Hz stimulation condition in order to study people with chronic paralysis (and subsequent osteoporosis) (McHenry and Shields 2012). Figure 4.2

illustrates an example of the force time curves, with the stimulation pulses, of one contraction using the 5 and 20 Hz stimulation protocols.

The 5 or 20 Hz electrical stimulation intervention consisted of 20 bouts of 50 contractions. Each contraction consisted of 10 stimulus pulses delivered at a frequency of either 5 Hz or 20 Hz. A work-to-rest ratio of one on and two off was maintained for both stimulation protocols. The total number of stimulus pulses (10,000) and relative time between contractions was identical between the 5 Hz and 20 Hz protocols. The 5 Hz stimulation session (1.6 hours) was 4 times longer than the 20 Hz stimulation session (0.42 hours). A 1-minute rest followed each bout of 50 contractions.

*Muscle Biopsy and Exon Microarray Protocol*

A subset of subjects (n=6) underwent bilateral percutaneous muscle biopsies 3 hours after the 20 Hz and 5 Hz electrical stimulation protocol on separate days (Figure 4.1CD) . We normalized all gene expression signals from the stimulated leg to the same subjects opposite leg muscle that did not receive the stimulation on that day (20 Hz or 5 Hz). Upon return at least 1 month later, the control leg from the first session received the stimulation protocol (20 Hz or 5 Hz) and the opposite leg served as the control. The leg that received the stimulation was always biopsied first followed by the control limb that did not receive stimulation.

We have described in detail the biopsy procedure in a previous report (Adams et al. 2011). Briefly, we took percutaneous muscle biopsies from both the intervention and control vastus lateralis muscle of each subject using a Temno biopsy needle (T1420, Cardinal Health) under ultrasound guidance within a sterile field. Five passes of the needle were made to assure a wide sampling range within the muscle. Each pass of the

needle was through the same incision site, but we altered the needle angle to sample a different part of the muscle. Because of severe muscle atrophy, each pass generated less than ~ 20 mg of muscle tissue. Following harvest, we placed the muscle biopsy samples in RNALater (Ambion) and stored at -80 °C until further use.

The RNA extraction procedure has been previously described (Petrie et al. 2014a). Briefly, RNA was extracted using the RNEasy Fibrous Tissue Kit (Qiagen) with DNAse to remove genomic DNA from final samples. Microarray hybridizations were performed at the University of Iowa DNA Facility as previously reported (Adams et al. 2011; Petrie et al. 2014a). Briefly, 50 ng total RNA was converted to SPIA amplified cDNA using the WT-Ovation Pico RNA Amplification System, v1 (NuGEN Technologies, San Carlos, CA, Cat.#3300) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN MinElute Reaction Cleanup column (QIAGEN Cat #28204) according to modifications from NuGEN. Four µg of SPIA amplified DNA were used to generate ST-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies, Cat #2000) and again cleaned up with the Qiagen column as above. 5µg of this product were fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module, v2 (NuGEN Technologies, Cat. #4200) as per the manufacturer's recommended protocol. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Human Exon 1.0 ST arrays (Part No. 900650), and incubated at 45º C for 18 h with 60 RPM rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), signal amplified with anti-streptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix

Model 3000 scanner with 7G upgrade and data were collected using the GeneChip operating software (GCOS) v1.4. All microarray data are MIAME compliant and have been submitted to the Gene Expression Omnibus (accession number pending).

*Exon Microarray Analysis*

The Affymetrix Human Exon 1.0 ST arrays were normalized using a Robust Multi-array Average (RMA) and transformed into a log2 hybridization signal using Partek Genomic Suites (v6.6 Copyright © 2013 Partek Inc., St. Louis, MO, USA). The log2 hybridization signals were analyzed using an expression profiling technique. All mRNA transcripts with log2 hybridization signals less than 2 standard deviations below the mean signal intensity for all subjects were discarded from the analysis, restricting the analysis to only those mRNA transcripts with high signal relative to background. A paired sample t-test was used to find mRNA transcripts with a significant difference between the stimulated and non-stimulated limb with a stimulation frequency. All mRNA transcripts with a p-value less than 0.05 were used to assess fold change differences. The fold change was calculated by subtracting the log2 hybridization intensity of the non-stimulated control limb from the log2 hybridization intensity of the stimulated limb. Any mRNA transcripts with a mean FC greater than 2.0 or less than 0.667 were considered differentially expressed. If the FC was less than 1, the negative inverse of the FC is reported to indicate a gene is less expressed in the stimulated limb compared to the non-stimulated limb.

*qPCR Procedure and Analysis*

We homogenized the muscle samples in lysis buffer using a tissue homogenizer and hard tissue grinding tip (Omni) followed by a column-based RNA extraction using the RNEasy Fibrous Tissue Kit (Qiagen). We included DNAse to ensure absence of

genomic DNA in final samples then eluted the RNA samples in water and quantified via nanodrop method then assayed each sample using the Agilent 2100 Bioanalyzer and reverse transcribed the high quality RNA samples iScript supermix (Bio-Rad). We standardized all input quantity of RNA for all reactions (500 ng each). At the time of qPCR plate preparation, we diluted the cDNA samples five-fold in water and analyzed via SYBR green technology using a custom PrimePCR plate (Bio-Rad) followed by an analysis in duplicate at a final concentration of .5 ng/µL cDNA per 10µL reaction using an ABI 7900 machine. We converted all expression levels to a relative fold change (FC) of the acutely stimulated limb to the control limb using the comparative $C_T$ method. Beta 2 macroglobulin (B2M) served as the reference gene because it demonstrated a consistent expression across all limbs. We validated with qPCR on a subset of genes including NR4A3, ABRA, and MSTN.

*Statistical Analysis*

We used a two-way analysis of variance with repeated measures to compare the maximum twitch force generated before and after the 5 Hz and 20 Hz exercise. Stimulation frequency (5 Hz or 20 Hz) and time were independent factors. We used the Tukey procedure for all post hoc analysis. We used a t-test to determine if the intervention increased or decreased gene expression. We used regression analysis to calculate coefficients of determination (R-squared) to assess any relationships between fatigue and gene regulation. An alpha level of 0.05 was used to test for significance. Data are reported as mean and standard deviation (mean±sd).

## Results

*Effect of stimulation protocol on muscle fatigue*

The peak muscle force for the cohort was 2.68±0.27 times higher for the 20 Hz stimulation as compared to the 5 Hz stimulation ($p<0.001$). The peak single twitch forces before and after the 20 Hz sessions were 30.92±5.52N and 12.22±4.13N, respectively ($p<0.001$); while the peak single twitch forces before and after the 5 Hz sessions were 27.58±4.42N and 12.06±3.35N ($p<0.001$), respectively (Figure 4.3). The magnitude of fatigue was not different between the two stimulation frequencies ($p=0.31$), supporting that a low-force stimulation protocol (5 Hz), distributed over time (1.6 hours), induced a similar level of muscle fatigue to that of a high-force protocol (20 Hz) delivered in a short time (0.42 hours). Importantly, the number of pulses (energy) delivered to the muscle was constant.

*Highest Regulated Gene Expression after 5 or 20 Hz stimulation*

We analyzed over 17,000 genes 3 hours after a session of 5Hz and 20Hz neuromuscular electrical stimulation. 1,084 and 748 genes had unadjusted p-values below 0.05 for the 5Hz and 20Hz training groups, respectively. Of those, 29 were up regulated with a fold change greater than 2.0 and 66 were down regulated with a fold change less than -1.5 in the 5 Hz group. In contrast, 48 were up regulated with a fold change greater than 2.0 and 16 were down regulated with a fold change less than -1.5. Seventeen genes were up regulated in both groups with NR4A3, PGC-1α, EGR1, ABRA, and GEM in the top 10 most up regulated genes in both groups. The fold-change for NR4A3, PGC-1A, EGR1, ABRA, and GEM was 12.40±2.93, 5.09±0.71, 4.87±2.11, 4.56±0.87, and 3.24±0.81, respectively, after 5Hz muscle stimulation. The fold-change for NR4A3, PGC-1A, EGR1, ABRA, and GEM was 9.72±2.79, 4.97±1.44, 9.09±3.61,

3.51±0.66, and 5.77±2.52, respectively, after 20 Hz muscle stimulation. There was no significant difference in the fold change between the 5 Hz and 20 Hz exercise sessions for NR4A3, PGC-1α, EGR1, ABRA, and GEM (p=0.513, p=0.948, p=0.553, p=0.285, p=0.238). Two genes (ABCA10 and TXNIP) were the only down regulated genes common to both groups.

*Metabolic Transcription and Fiber type Gene Regulation*

We investigated the influence of 20 Hz and 5 Hz stimulation on a subset of genes typical of a diabetic phenotype. As described above, there was a significant increase in the expression of major muscle metabolic transcription factors including PGC-1α, NR4A3, and ABRA, and a significant decrease in the expression of MSTN after 20 Hz and 5 Hz stimulation (Figure 4.4A-D); however, there was no difference between the 20 Hz and 5 Hz condition. This finding is congruent with the similar physiological level of fatigue induced by each protocol, despite 40% lower force generated by the 5 Hz protocol. The acute bout of stimulation did not regulate the fast and slow twitch genes for either the 20 Hz or 5 Hz condition (Figure 4.4E-L).

*Glycolytic and Fatty Acid Metabolism Gene Regulation*

Both the 20 Hz and 5 Hz condition showed a trend for an increased PDK4 expression following the stimulation, however only the 5 Hz condition was significant ($p<0.05$) (Figure 4.5A). There was a significant decrease in PDHA1 but only after the 5 Hz stimulation condition (Figure 4.5B). There were no differences detected in any genes associated with fatty acid oxidation.

*Oxidative Phosphorylation, and Mitochondria Gene Regulation*

There were no differences detected in gene expression after 20 Hz or 5 Hz stimulation for the tricarboxylic acid cycle and oxidative phosphorylation genes (Figure 4.6); however, there was a trend for a decrease in the expression of COQ10A (p=0.087), but only after 20 Hz stimulation (Figure 4.6H). MFN2 gene was repressed after 20Hz stimulation (p=0.038), but was unchanged after 5 Hz stimulation (Figure 4.6L). MFF gene was repressed after 5 Hz (p=0.021), but was unchanged after 20 Hz (Figure 4.6K). There were no other mitochondrial remodeling genes modulated by the 20Hz and 5Hz training (Figure 4.6I-K).

*Relationship between Gene Expression and fatigue*

In an effort to understand if the amount of fatigue among subjects was able to explain the magnitude of change in gene expression, we assessed the relationship between the change in twitch force (fatigue) and the change in gene expression. The coefficients of determination were 0.29, 0.03, 0.10, and 0.69 for PGC-1α, NR4A3, MSTN, and ABRA, respectively. The fatigue after the 20 Hz and 5 Hz protocol was internally consistent among all subjects (Figure 4.3).

*qPCR Validation*

We analyzed a subset of representative genes that regulate metabolism, hypertrophy, and atrophy. In all cases, the qPCR analysis confirmed the microarray effects, albeit, the fold-changes were greater with qPCR. There was a 12.4±2.4 and 200.9±50.4 fold-change for NR4A3, 6.0±0.4 and 21.6±6.3 fold-change for ABRA, and 0.56±0.06 and 0.42±0.2 fold-change for MSTN. No differences in gene expression were detected between the 5 Hz and 20 Hz condition using qPCR analysis supporting the results obtained from the microarray analysis.

## Discussion

We examined the effect of low (5 Hz) and high (20 Hz) frequency stimulation on muscle fatigue and mRNA expression in human paralyzed muscle. The objective of the study was to determine if low frequency stimulation, and the associated low-force, challenges skeletal muscle to trigger fatigue and the expression of key metabolic gene regulators. The low-force, 5 Hz stimulation protocol induced a similar amount of fatigue (examined using a 3 Hz single twitch assessment) as the 20 Hz protocol. Both stimulation protocols caused a robust up regulation of major muscle transcription factors associated with muscle metabolism (PGC-1α, NR4A3, and ABRA). This finding is important, because the 20 Hz protocol induced nearly 3 times more force than the 5 Hz protocol and suggests that a low-force protocol may be effective in regulating metabolism of skeletal muscle in people with a weakened skeletal system.

Skeletal muscle fibers are generally highly adaptable and capable of altering their metabolic and contractile properties based on the dose of physiological stress (exercise). A skeletal muscle that is regularly stressed will trigger the transformation from fast glycolytic muscle to oxidative, fatigue resistant muscle (Adams et al. 2011; Coffey and Hawley 2007; Dudley-Javoroski and Shields 2008b; Shields 1995; 2002). In the absence of regular physical activity, skeletal muscle becomes less able to metabolize sugar and fat, leading to a diabetic phenotype that contributes to an altered blood-glucose regulation pathway (Bjornholm and Zierath 2005; Stuart et al. 2013b). Not surprisingly, individuals with chronic spinal cord injury commonly develop insulin resistance and diabetes (Duckworth et al. 1983; Duckworth et al. 1980) and a muscle phenotype that resembles that of non- paralyzed people with diabetes (Stuart et al. 2013b). It is logical that people with spinal cord injury may promote improved systemic metabolic health by regular use of their paralyzed musculature; however, the strategy

used to activate the muscle is complex because of the muscle force applied to the weakened skeletal system after spinal cord injury. The important finding from this study is that major metabolic gene transcription pathways are regulated at low-force levels as compared to high-force levels (5 Hz and 20 Hz).

Skeletal muscle operates at a mechanical disadvantage within the human body. In order to offset the high external torques from gravity, skeletal muscle must generate high-force when moving a limb against gravity. When we consider the limited length of the anatomical lever arm for muscle, it is common for a muscle force to be in excess of 6 times body weight, a stress that can damage osteoporotic bone (Hartkopp et al. 1998). We have previously quantified that people with spinal cord injury lose between 2 and 4 percent of their bone each month after a spinal cord injury (Dudley-Javoroski and Shields 2008b); and understand that a fracture can be life threatening for people with chronic spinal cord injury (Carbone et al. 2013). As we search for methods to normalize the metabolic state through skeletal muscle activity, we realize that the force should be low to protect the underlying bone. The findings of this study support that a low-force exercise, induced through a low frequency electrical stimulation, is effective at physiologically challenging the skeletal muscle (fatigue), and increasing the expression of several stress response genes, specifically PGC-1α, NR4A3, and ABRA. These genes play a key role in the initiation of transcription and translation of proteins required for oxidative metabolism and are known to induce long-term oxidative muscle adaptations (Chan and Arany 2014; Finck and Kelly 2006; Kang and Li Ji 2012; Mootha et al. 2003).

Exercise is a well-known stimulus of PGC-1α expression in skeletal muscle (Edgett et al. 2013; Egan et al. 2010; Little et al. 2011b); however, there is a knowledge gap about the

actual dose of muscle force necessary to optimally regulate this gene. Previous reports support that high intensity exercise increases PGC-1α expression (Edgett et al. 2013; Egan et al. 2010; Little et al. 2011b) to a greater extent than lower intensity exercise (Edgett et al. 2013; Little et al. 2011b). However, these studies generally measure the intensity of whole body exercise while on a stationary bicycle using a percentage of the synergistic muscle workload (oxygen consumption). An inverse relationship between PGC-1α expression and whole body high intensity exercise, as recently reported, supports there may be methodological issues, like incomplete muscle fiber recruitment, affecting the biopsy sample in humans (Edgett et al. 2013). Because we control the input to the vastus lateralis through supra maximal electrical stimulation, we were confident that nearly every fiber was activated, reducing the risk of us sampling muscle that had not been exercised. We also delivered the identical number of stimulation pulses (10,000), but manipulated the duration of the exercise (time). By positioning the limb in a less than optimal length (knee extension) we could generate a differential force between the 20 Hz and 5 Hz condition, but at an acceptable stress for people with a fragile skeletal system. To our knowledge, no previous studies have directly evaluated a low-force (long duration) and high-force (shorter duration) session of exercise with a controlled stimulation intensity (supra maximal) and pulse number on gene expression in human skeletal muscle.

In people without spinal cord injury, a long-duration, low intensity voluntary exercise performed throughout the day caused an improved sensitivity to insulin as compared to a high-intensity exercise even at similar levels of energy expenditure (Bankoski et al. 2011; Dunstan et al. 2012; Duvivier et al. 2013). We designed this study using several important translational principles in order to assess the effect of a low-force to a high-force muscle contraction on gene regulation. First, we reduced the total shear force of

the muscle on the skeletal system by extending the knee and by placing the quadriceps in a less than optimal length (McHenry and Shields 2012). Second, we preserved a force differential between the 20 Hz condition and the 5 Hz condition in the knee extension position. On average, the 5 Hz condition generated around 40% of the force produced at 20 Hz; although both muscle forces were nearly 25% lower with the knee in the more extended position (as compared to the 90° flexed position in pilot data from our lab). Third, we maximally activated nearly all fibers of the muscle to eliminate any issue of biopsy sampling error from incomplete muscle fiber recruitment. Finally, we discovered that we induced a similar level of muscle fatigue when we delivered a long duration low-force exercise compared to a high-force shorter duration exercise. Taken together, these findings are novel because they represent the first study to control for several factors that are essential to understanding gene regulation in human muscle. However, these findings are not without important methodological considerations. For example, we used just one time point (3 hours) to assess the effects on gene regulation; an approach necessary to limit tissue injury in people with spinal cord injury, but limiting our ability to generalize these findings to other time points.

In people without spinal cord injury, muscle fibers are recruited only as they are needed to complete a given task (Bawa et al. 1984; Binder et al. 1983; Henneman 1985). Low intensity exercise requires fewer muscle fibers, whereas high intensity exercise recruits nearly all muscle fibers. Because fewer muscle fibers are active during low recruitment exercise, the ratio of activated to non-activated muscle fibers is often uncertain in the absence of EMG recordings. Therefore, the high intensity exercise and subsequent full muscle recruitment typically increases the probability that biopsied fibers will demonstrate the response to exercise. Because we maximally activated the entire muscle at a low frequency (low-force), we were able to examine a novel “physiological

condition" that does not occur during voluntary activation of human muscle. Indeed, nearly 100% recruitment of a muscle at a low-force is novel and enabled us to address the question of whether peak force is an integral part of regulating these major metabolic gene transcription pathways. Our findings suggest that low-force over a long duration is a robust modulator of these metabolic genes; a new finding that has important implications today for people with spinal cord injury.

The absolute mechanism contributing to the signaling of these pathways remains elusive. Calcium release and sequestration within a muscle fiber determines the number of cross-bridges activated and force developed during a muscle contraction. The CAMKII pathway, which is triggered by calcium, is thought to signal the transcription and translation of PGC-1α and other metabolic regulators (Chin 2004; Egan et al. 2010; Kramerova et al. 2012; Rose et al. 2006). However, the mechanical transduction stimulus has also been hypothesized to be essential to trigger muscle signaling pathways (Goldspink 2003), perhaps from the drop in ATP and increase of AMP (Freyssenet 2007). In this study, we controlled the amount of energy delivered to the muscle between the low and high-force exercises. The number of stimulus pulses delivered during each condition was constant; therefore, the number of times the muscle released calcium was consistent. The major difference between the 5 Hz and 20 Hz conditions was the time required to deliver the stimulus pulses and the resulting contractile force. Taken together, our results support that the mechanical load differential delivered in this protocol had a minimal influence on regulating the metabolic gene transcription factors in paralyzed muscle.

*Summary and Conclusions.*

Oxidative skeletal muscle is a critical tissue for systemic metabolic health. After a spinal cord injury, oxidative skeletal muscle transforms into a glycolytic muscle consistent with a diabetic phenotype and the skeletal system becomes osteoporotic within the first year after injury. We demonstrated that a low muscle force exercise caused significant muscle fatigue and a robust increase in the expression of PGC-1α and other metabolic transcription factors that transform skeletal muscle into an oxidative phenotype. The findings from this study hold promise that this intervention may be an effective training protocol that does not increase risk of bone injury in people suffering with chronic spinal cord injury. Future studies are also underway to understand the full impact of daily low-force muscle training on overall systemic metabolic health in people with paralysis.

## Table

**Table 4.1 Chapter 4 subject characteristics**

| Subject | Injury Level | Years Post Injury |
|---|---|---|
| 01 | T4 | 11 |
| 02 | C4-5 | 2 |
| 03 | C4-6 | 1 |
| 04 | T5-7 | 9 |
| 05 | T6-7 | 9 |
| 06 | T9-10 | 13 |
| 07* | T8 | 5 |
| 08* | T8 | 1 |
| 09* | T4 | 3 |
| 10* | T8 | 3 |
| 11* | T4 | 5 |
| 12* | T10 | 5 |

* Biopsy of stimulated and control Leg after 5 and 20 Hz session

## Figures

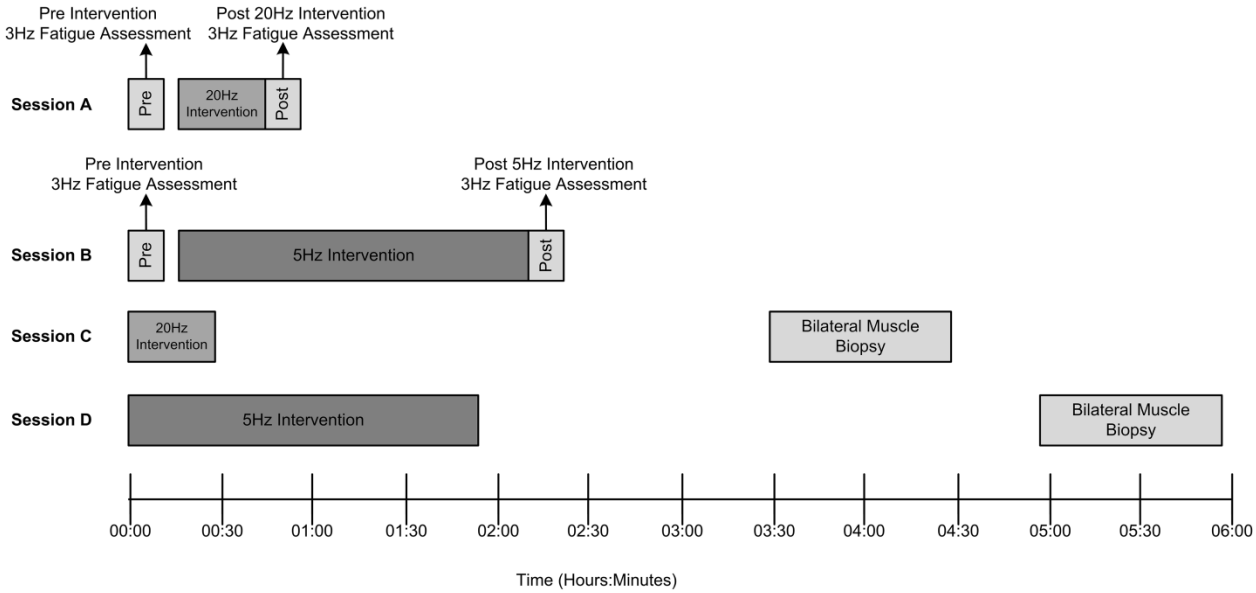


**Figure 4.1 Timeline for each experimental session**
(A, B): In the first two sessions, the physiological response to a 5 Hz or 20 Hz stimulation protocol was assessed using pre and post 3 Hz stimulation. These first two sessions (A, B) were separated by at least two weeks. (C, D): In the second two sessions, bilateral vastus lateralis muscle biopsies were taken 3 hours after unilateral activation of the vastus lateralis (quadriceps) muscle with the 5 Hz or 20 Hz stimulation protocol. These last two sessions (C, D) were separated by at least 6 weeks.

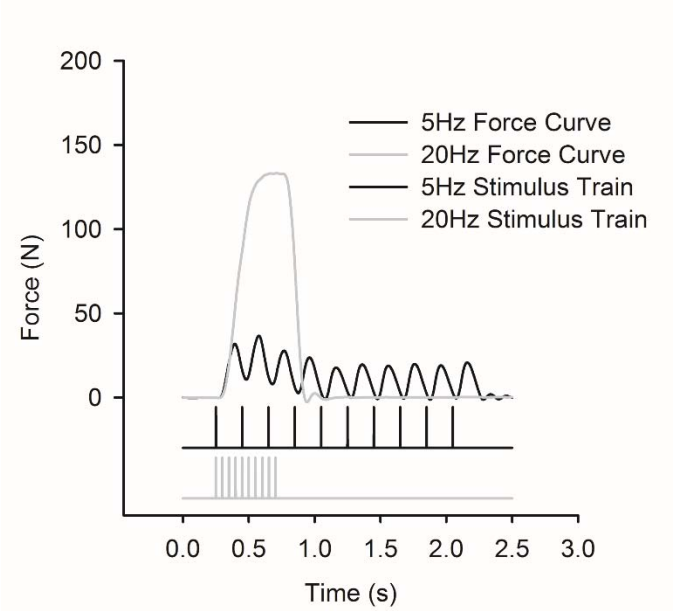


**Figure 4.2 Representative example of the force-time curve**
An example of the force generated using 5 Hz (black) and 20 Hz (grey) neuromuscular electrical stimulation with the knee extended to 10° of horizontal in a single subject. The associated electrical stimulation train is below each force-time curve.

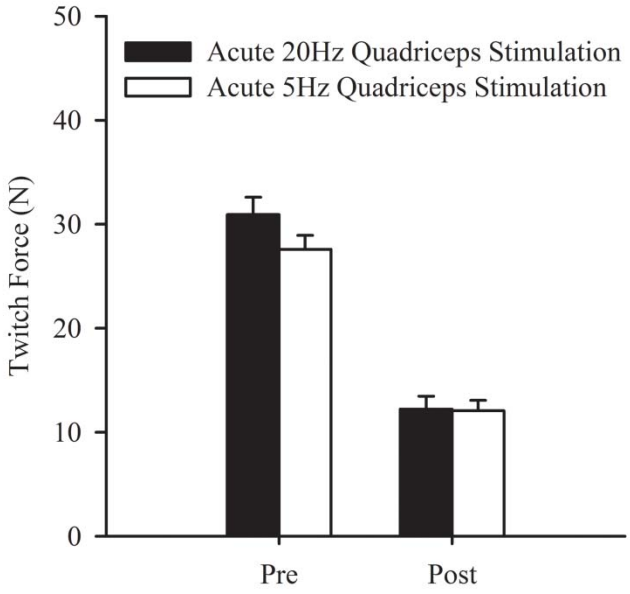


**Figure 4.3 Assessment of fatigue after low and high-force exercise**
The mean and standard error of the maximum twitch force generated before (pre) and after (post) a session of low (5 Hz) and high frequency (20 Hz) exercise using electrical stimulation. There was a significant decrease in the peak twitch force after low ($p<0.001$) and high ($p<0.001$) force exercise. There was no difference in the peak twitch force observed between the low and high-force exercise sessions ($p=0.31$).

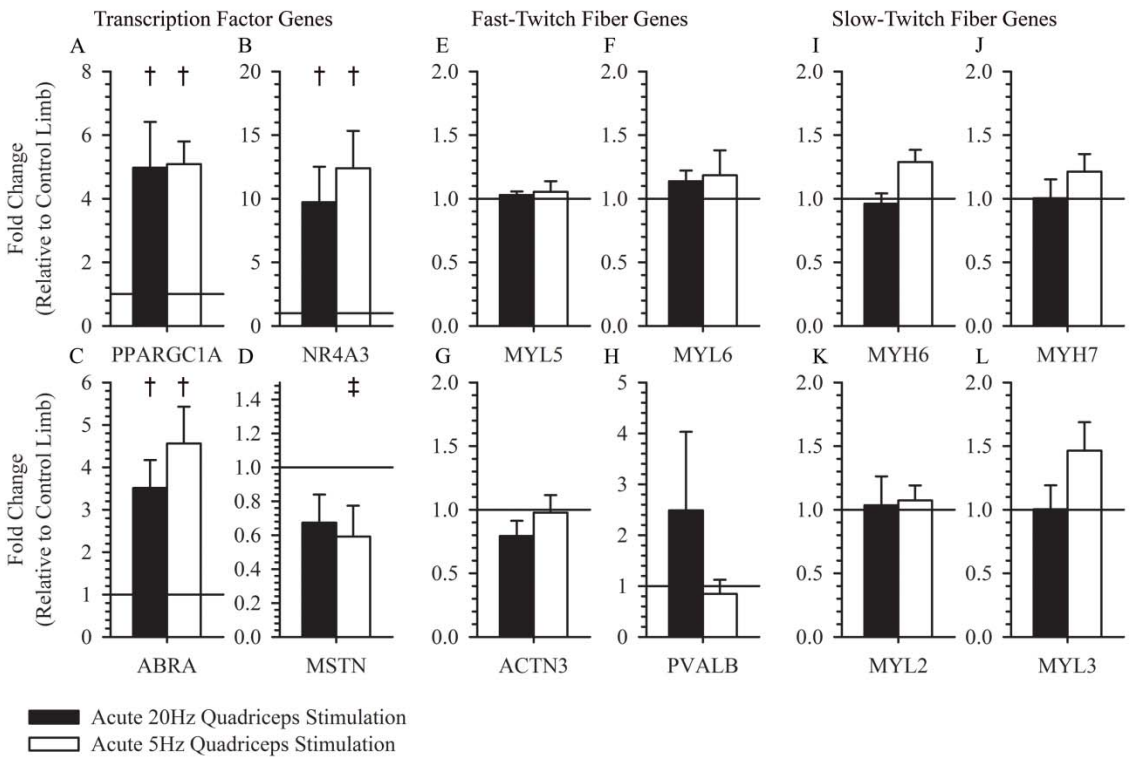


**Figure 4.4 Expression of transcription factor, fast-twitch fiber, and slow-twitch fiber genes following acute neuromuscular electrical stimulation**
(A) PGC-1α was increased 3 hours after a dose of low and high frequency muscle stimulation ($p<0.001$ and $p=0.003$, respectively). (B) NR4A3 was increased 3 hours after a dose of low and high frequency muscle stimulation ($p<0.001$ and $p<0.001$, respectively). (C) ABRA was increased 3 hours after a dose of low and high frequency muscle stimulation ($p=0.001$ and $p=0.003$, respectively). (D). MSTN was decreased 3 hours after a dose of low frequency but not high frequency muscle stimulation ($p=0.05$ and $p=.11$, respectively). (E-H) Genes associated with a fast-twitch muscle fiber phenotype were unaltered 3-hours after either low or high frequency muscle stimulation. In a single subject, PVALB was increased after neuromuscular electrical stimulation but this was inconsistent with the remaining subjects. (I-J) Genes associated with a slow-twitch muscle fiber phenotype were unaltered 3-hours after either low or high frequency muscle stimulation. Fold change values represent the mean of the exercised limb compared to the opposite (non-exercised) limb from the same subjects. † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

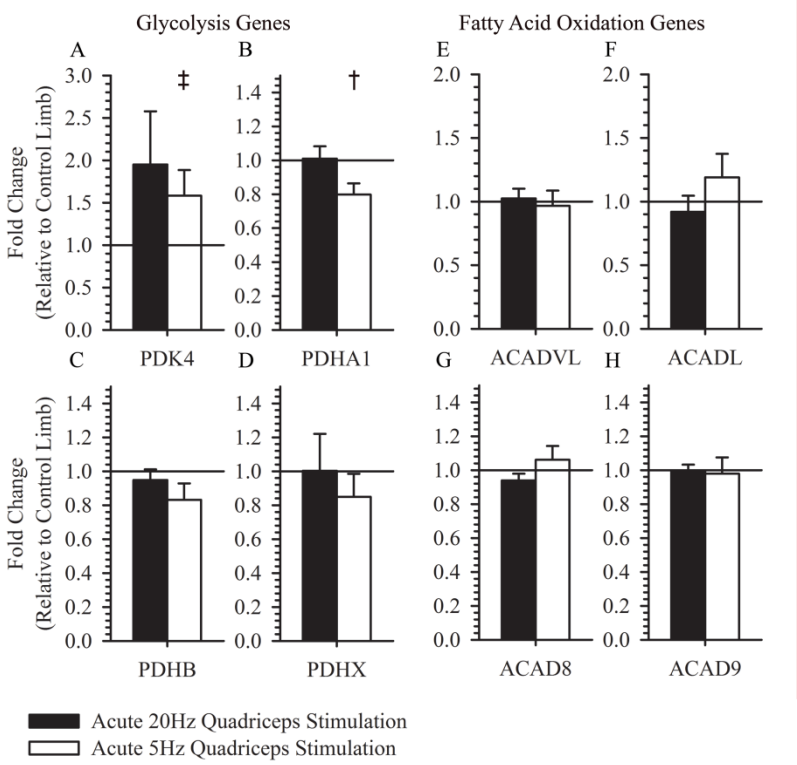


**Figure 4.5 Expression of glycolysis and fatty acid oxidation genes following acute neuromuscular electrical stimulation**

(A-D) Genes associated with glucose metabolism were consistently unaltered 3 hours after low or high frequency muscle stimulation. However, PDK4 appears to be increased in most subjects after both low and high frequency muscle stimulation, PDHA1 decreased after low frequency muscle stimulation. (E-H) Genes associated with fatty acid metabolism were unaltered 3-hours after either low or high frequency muscle stimulation. Fold change values represent the mean of the exercised limb compared to the opposite (non-exercised) limb from the same subjects. † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

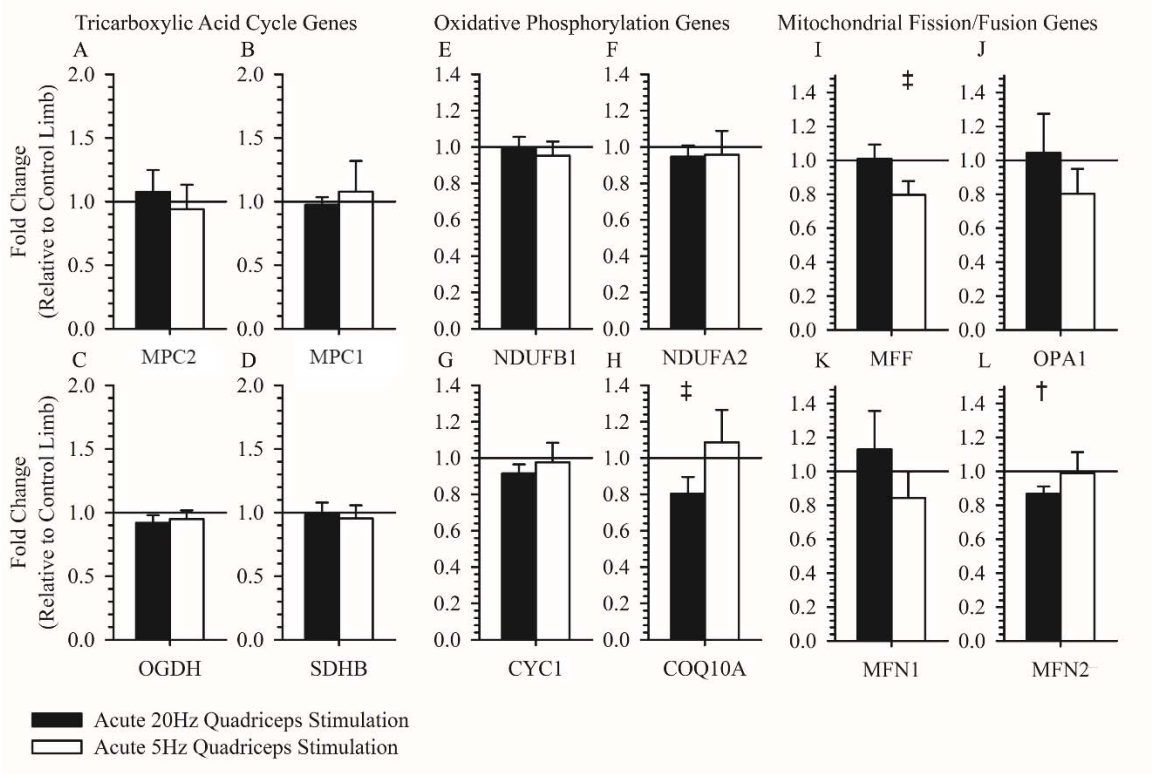


**Figure 4.6 Expression of tricarboxylic acid cycle, oxidative phosphorylation, and mitochondrial fission/fusion genes following acute neuromuscular electrical stimulation**
(A-D) Genes associated with the tricarboxylic acid cycle were unaltered 3 hours after low and high frequency muscle stimulation. (E-H) Most genes associated with oxidative phosphorylation were unaltered 3 hours after either low or high frequency muscle stimulation; however, COQ10A was decreased after high but not low frequency neuromuscular electrical stimulation. (I-J) Most genes associated with the mitochondrial fission or fusion were unaltered 3 hours after either low or high frequency muscle stimulation. However, MFF was decreased in most subjects after low frequency muscle stimulation and MFN2 was decreased in most subjects after high frequency muscle stimulation. Fold change values represent the mean of the exercised limb compared to the opposite (non-exercised) limb from the same subjects. † indicates a p-value < 0.05 for a within group paired t-test. ‡ indicates a p-value < 0.10 for a within group paired t-test.

# CHAPTER 5 DOSE ESTIMATE OF LOW-FORCE EXERCISE NEEDED TO ALTER GENE EXPRESSION IN PARALYZED HUMAN SKELETAL MUSCLE

## Introduction

The loss of physical activity after a spinal cord injury culminates in severe musculoskeletal deterioration that compromises the health of people with paralysis. Paralyzed skeletal muscle atrophies and transforms into a glycolytic, highly fatigable phenotype (Dudley-Javoroski and Shields 2008b; Shields 1995; Shields and Chang 1997; Shields and Dudley-Javoroski 2006). The underlying bone becomes severely osteoporotic with a high susceptibility to fracture, even during routine activities (Dudley-Javoroski et al. 2013; Dudley-Javoroski et al. 2012; Dudley-Javoroski and Shields 2013; 2008b; 2012). We previously reported that high-force exercise training using muscle stimulation reduces musculoskeletal deterioration when an intervention is started soon after a spinal cord injury (Adams et al. 2011; Dudley-Javoroski et al. 2013; Dudley-Javoroski et al. 2012; Dudley-Javoroski and Shields 2013; 2012; Petrie et al. 2014b; Shields and Dudley-Javoroski 2007; 2006). However, recent evidence supports that long duration, low-force activity may have an important impact on skeletal muscle metabolism (Chau et al. 2013; Dunstan et al. 2012; Healy et al. 2008; Nguyen et al. 2011) in people with osteoporotic limbs, but how to optimally dose low-force exercise has not been elucidated.

Low-force exercises using neuromuscular electrical stimulation are unique because they are thought to recruit muscle fibers differently from volitional contractions (Bickel et al.

2011; Cooper 1930). Neuromuscular electrical stimulation depolarizes alpha motor neuron axons to evoke muscle contractions. Each stimulus pulse triggers the release of calcium from the sarcoplasmic reticulum resulting in a muscle contraction. When stimulus pulses are widely spaced, an interpulse interval of >200ms (<5Hz stimulation frequency), the muscle releases and fully sequesters calcium for each stimulus pulse resulting in a train of unfused muscle twitches rather than a fused tetany. Decreasing the interpulse interval (high frequency) prevents the full sequestration of calcium, which results in the full fusion of muscle twitches and an overall increase in contraction force (Bickel et al. 2011; Cooper 1930). In people with chronic paralysis, the underlying bone is at risk for fracture during these high-force contractions, particularly when performed in the seated position (McHenry and Shields 2012). Conversely, low-frequency induced contractions provide an alternative intervention to increase muscle activity without generating a high-force. However, the optimal number of days per week to train or the number of stimulus pulses delivered per training session has not been explored. In our previous work, we reported that a single acute bout of low frequency (low-force) exercise induced key metabolic transcription factors, like peroxisomal proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) (Petrie et al. 2015; Petrie et al. 2014b), suggesting that a repetitive training program would induce long term adaptations in both the genotype and phenotype of paralyzed muscle. Before a full scale randomized controlled clinical trial is carried out, we need to understand the optimal amount of stimulation that is necessary to induce changes in muscle fatigue and baseline gene regulation. To our knowledge, no empirical data exists to estimate the optimal dose of low frequency stimulation needed to be incorporated into a full blown clinical trial.

Our primary goal in this study was to explore the relationship between dose (sessions per week and number of stimulus pulses) and skeletal muscle genotype and phenotype

after training with a low-force exercise intervention. We expected that there would be a strong positive correlation with the number of training doses performed per week and the resulting change in fatigability and gene expression profile. Based on the results of this study we will have empirical evidence to support doses of low frequency exercise used in future clinical trials.

## Methods

*Subjects*

Seven people with a complete spinal cord injury (ASIA-A) at least 1-year duration participated in this pre-clinical study (Table 5.1). Subjects unilaterally trained the quadriceps muscle with our low-force exercise for at least 12 weeks while keeping the other limb untrained. Before and after training, subjects performed a muscle performance assessment to evaluate muscle potentiation and fatigue. After training, subjects underwent a percutaneous muscle biopsy of the trained and untrained limb. People with a history of lower limb fracture, peripheral nerve injury, pressure ulcers, or history of muscle injury will be excluded or not allowed to continue with the study. Written consent approved by the University of Iowa Institutional Review Board was obtained for all participants.

*Muscle Performance Assessment*

The muscle performance assessment consisted of a repetitive muscle twitch exercise using a 3Hz stimulation frequency as previously described (Petrie et al. 2014a; b). Briefly, the assessment consisted of 2 trains of supra-maximal Neuromuscular electrical stimulation delivered to the quadriceps muscle with the lower limb secured and the ankle fastened to a force transducer. Participants sat with the hip and knee at 90° and the

lower leg secured to a padded, semicircular metal plate just above the ankle. Two self-adhesive 7cm x 13cm oval carbon electrodes (EMPI, Inc. St. Paul, MN) were adhered to the skin over the quadriceps muscles' motor point and connected to a constant current electrical muscle stimulator unit (Digitimer model, DS7A, Digitimer, Welwyn Garden City, Hertfordshire, UK) under software control, as previously reported (Dudley-Javoroski et al. 2008; Petrie et al. 2014a; b). The stimulation intensity was set by slowly increasing the stimulus intensity using single pulses spaced 10 seconds apart. Supra-maximal intensity was verified by no increase in force development during the muscle twitch with subsequent increases in the stimulus intensity. Two trains of neuromuscular electrical stimulation delivered 1,000 and 200 stimulus pulses, respectively, using a 3Hz stimulation frequency. The resulting force developed during each muscle twitch was recorded using an analog-to-digital data acquisition board at a sample rate of 2,00Hz controlled by a custom Labview application (National Instruments). A 5-minute recovery period was given between the first and second train to allow for some muscle recovery.

*Training Protocol*

The low-force training protocol was performed in a seated position with the training limb extended at the knee. Self-adhesive 7cm x 13cm oval carbon electrodes (EMPI, Inc. St. Paul, MN) adhered to the skin over the quadriceps muscles' motor point, as previously described(Dudley-Javoroski et al. 2008; Petrie et al. 2015; Petrie et al. 2014a; b). Training occurred either in the laboratory using a constant current muscle stimulator (DS7A Digitimer, Digitimer, Welwyn Garden City, Hertfordshire, UK) or at home using a portable commercial muscle stimulator (EMPI Infinity Plus, EMPI, Inc. St. Paul, MN). The laboratory stimulator was set to deliver 200μs pulses at intensities up to 400mA to ensure supra-maximal training. The at-home muscle stimulator was set to deliver 450μs pulses at intensities up to 100mA. Due to federal regulations, current intensity is limited

to 100mA for the at-home training sessions. We increased the duration of each stimulus pulse to 450μs to increase the current density of each stimulus pulse and maximize muscle fiber recruitment. Most participants were able to supra-maximally trained during at-home training sessions. Participants were asked to train at least 3 days per week. Training compliance was monitored using an automated logging system in the at-home or laboratory stimulators. Subjects were asked to maintain a written log of all at-home training sessions, including time, date, and maximum intensity the stimulator reached.

Four subjects completed a low-force training protocol that consisted of 2 bouts of Neuromuscular electrical stimulation delivered at a 5Hz stimulation frequency. During each bout, 60 trains of 10 stimulus pulses at a 1 on to 2 off work rest ratio were delivered. Each train was 2-seconds in duration and was followed by 4-seconds of rest. Each bout took approximately 6 minutes to complete. A 5-minute rest period was given between the first and second bout, making the total training time to be 17 minutes. 1,200 stimulus pulses were delivered during each training session. Three subjects completed a low-force training protocol that consisted of 6 bouts of neuromuscular electrical stimulation. During each bout, 120 trains of 10 stimulus pulses were delivered using the same 1 on to 2 off work rest ratio. Each bout took approximately 12 minutes with a total training time of approximately 90 minutes. 6,000 pulses were delivered in each training session.

*Muscle Biopsy and Exon Microarray Procedure*

After training, subjects underwent percutaneous muscle biopsies of the trained and untrained vastus lateralis using a previously reported procedure (Adams et al. 2011; Petrie et al. 2014a; b). Briefly, percutaneous muscle biopsies were taken from both the trained and untrained vastus lateralis muscle of each subject using a Temno biopsy

needle (T1420, CardinalHealth) under ultrasound guidance within a sterile field. Four to five passes of the needle assures a wide sampling range within the muscle and provide adequate samples for gene expression analysis. Following harvest, muscle samples were immediately placed In RNALater (Ambion) and stored at -80 °C until further use.

RNA was extracted from muscle specimen using our previously reported protocol (Petrie et al. 2015; Petrie et al. 2014a). Briefly, RNA was extracted using the RNEasy Fibrous Tissue Kit (Qiagen) with DNAse to remove genomic DNA. Affymetrix Human Exon 1.0 ST microarray hybridizations were performed at the University of Iowa DNA Facility. Approximately 50 ng of total RNA was converted to SPIA amplified cDNA using the WT-Ovation Pico RNA Amplification System, v1 (NuGEN Technologies, San Carlos, CA, Cat.#3300) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN MinElute Reaction Cleanup column (QIAGEN Cat #28204) according to modifications from NuGEN. 4 µg of SPIA amplified DNA will be used to generate ST-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies, Cat #2000) and cleaned with the Qiagen column described above. 5 µg of this product will be fragmented (average fragment size = 85 bases) and labeled using the NuGEN FL-Ovation cDNA Biotin Module v2 (NuGEN Technologies, Cat. #4200) as per the manufacturer's recommended protocol. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Human Exon 1.0 ST arrays (Part No. 900650), and incubated at 45º C for 18 h with 60 RPM rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), and amplified with antistreptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics

Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade and collected using the GeneChip Operating Software (GCOS) v1.4.

*Exon Microarray Analysis*

Our exon microarray analysis was performed using our previously reported technique (Petrie et al. 2015; Petrie et al. 2014a; b). The Affymetrix Human Exon 1.0 ST arrays signal intensities were normalized using a Robust Multi-array Average (RMA) and transformed into a log2 hybridization signal using Partek Genomic Suites (v6.6 Copyright © 2013 Partek Inc., St. Louis, MO, USA). The log2 hybridization signals were analyzed using a gene-level expression technique. All genes with log2 hybridization signals less than 2 standard deviations below the mean signal intensity for all subjects were discarded, restricting the analysis to only those genes with high signal intensities. Two filters were used to determine the genes that are being differentially expressed in the trained limb compared to the untrained limb: 1) genes with a p-value less than 0.05 calculated using a paired sample t-test between the trained and control limb, and 2) genes with a fold-change (FC) greater than or less than 1.5 and 0.667, respectively. The FC for each gene was calculated by subtracting the log2 hybridization intensity of the stimulated (trained) limb from the log2 hybridization intensity of the non-stimulated (control) limb. We use the subject's opposite (control) limb for all comparisons, to minimize the influence of confounding variables, such as circadian rhythm, diet, etc. We also explored the expression changes of changes in an *a priori* panel of genes important for muscle hypertrophy, metabolism, and the transformation to an oxidative phenotype (Petrie et al. 2014b). We compared the observed changes in fatigue index to the change in gene expression of these key genes.

*Statistical Analysis*

A least squares linear regression analysis was performed to evaluate the relationship between dose (training times per week or number of stimulus pulses per week) and changes in muscle phenotype biomarkers (fatigability and gene expression). Further, we used a least squares linear regression to explore the relationship between changes in gene expression after training and changes in fatigability. All data were reported as group means and standard deviations (mean ± standard deviation), unless otherwise specified.

## Results

Four subjects (01-04) completed at least 12 weeks of low-force training with a dose of 1,200 stimulus pulses, while 3 subjects (05-07) completed the low-force training with a dose of 6,000 stimulus pulses. Overall, the participants trained for an average of 19.9±3.5 weeks. On average, people trained 3.5±1.5 times per week with a range of 1.8 to 6.3 times per week. Those that trained with 6,000 stimulus pulses, trained for a longer duration (23.0±1.7 weeks) and more frequently (4.7±1.7 sessions/week) compared to those that trained with 1,200 stimulus pulses (17.5±2.4 weeks; 2.6±0.5 sessions/week).

*Muscle Performance Assessment*

A representative force curve (subject 05) from the muscle force assessment before and after training is shown in Figure 5.1. As expected, the muscle performance test using a train of stimulus pulses delivered 333ms apart resulted in a train of twitch-like contractions with a significant loss in force development at the end of the train. However, not all subjects responded equally. Overall, the mean fatigue index before training in the control and trained limb was 0.31±0.10 and 0.26±0.07, respectively. After training, the mean fatigue index in the control and trained limb was 0.27±0.09 and 0.39±0.2,

respectively (Figure 5.2). We used a linear regression model to explore the relationship between the change in fatigue index and the factors that define dose (training sessions per week and stimulus pulses per session). The number of training sessions per week had the strongest association to the change in fatigue index after training ($R^2$=0.81, $p<0.006$). We found significant colinearity between the number of training sessions per week and the number of stimulus pulses per session when used in a single model because the subjects that trained most frequently used the high 6,000 stimulus pulse protocol.

*Gene Expression after Training*

Fourteen trained and control muscle specimens were collected and analyzed from 7 people. Each trained limb's gene expression profile was normalized to the person's opposite (untrained) limb. We analyzed over 17,000 genes and found 228 genes were changed after at least 12 weeks of low-force training. Of those genes, 153 genes had a fold-change greater than 1.5 and 75 genes had a fold-change less than 0.67. None of the genes with the highest or lowest mean expression changes had direct associations with skeletal muscle. Further, few of the *a priori* genes described in our diabetic phenotype demonstrated consistent changes after training. We found a positive relationship between the number of training sessions performed per week and the expression of mitochondrial pyruvate carrier 1 (MPC1, $R^2$=0.51), mitochondrial pyruvate carrier 2 (MPC2, $R^2$=0.33), and myosin heavy chain 2 (MYH2, $R^2$=0.68). We also found a negative correlation between the fatigue index and expression of ACTN3 ($R^2$=0.21).

*Relationship between Muscle Performance and Gene Expression*

We explored the relationship between changes in the fatigue index after training to changes in gene expression. We found a positive relationship between the change in

fatigue index after training and the expression of mitochondrial pyruvate carrier 1 (MPC1, $R^2$=0.63), mitochondrial pyruvate carrier 2 (MPC2, $R^2$=0.50), and myosin heavy chain 2 (MYH2, $R^2$=0.89) (Figure 5.3A-C). We also found a negative correlation between the fatigue index and expression of ACTN3 ($R^2$=0.35) (Figure 5.3D). MPC1 is a crucial transport protein involved in shuttling pyruvate into the mitochondria for oxidation from the cytosol. Because of the importance of MPC1 in glucose metabolism, we also explored the relationship between the expression of MPC1 after training compared to other key metabolic genes (MPC2, PGC-1α, MYH2, and ACTN3). We found a strong positive correlation between MPC1 and MPC2 ($R^2$=0.79), PGC-1α ($R^2$=0.74), and MYH2 ($R^2$=0.86), while there was a negative correlation between MPC1 and ACTN3 ($R^2$=0.25) (Figure 5.4).

## Discussion

In this pre-clinical study, we explored the dose-response of low-force exercise training in people with long-standing paralysis. We unilaterally trained people with a spinal cord injury for at least 12-weeks with a low-force exercise evoked by neuromuscular electrical stimulation. We found a significant positive relationship between the number of training sessions performed in a week and the change in fatigue index after training. Additionally, there were strong associations between the change in fatigue and the expression of genes related to metabolic proteins (MPC1 and MPC2) and contractile proteins (MYH2 and ACTN3).These estimates will assist future work to determine how low-force exercise influences the metabolic health of people with a spinal cord injury.

We previously demonstrated that the transformation of paralyzed human skeletal muscle occurs from a fatigue-resistant phenotype to a highly fatigable phenotype within the first year (Dudley-Javoroski and Shields 2008b; 2012; Shields 1995). Exercise training using

neuromuscular electrical stimulation yielding high-force contractions sustains bone architecture and muscle fatigue resistance when started soon after an injury (Dudley-Javoroski and Shields 2013; 2008a; Shields and Dudley-Javoroski 2007; 2006; Shields et al. 2006b). However, few people with a complete spinal cord injury are prescribed routine exercise using neuromuscular electrical stimulation in clinical practice. Currently, musculoskeletal deterioration and the increased incidence of bone fractures, cardiovascular disease, and metabolic syndrome are common after spinal cord injury (Banerjea et al. 2008). Our previous work using a high-force exercise intervention would not be safe for people with long-standing paralysis. The high-force muscle contractions would place dangerous shear forces through bone that is at fracture threshold (Hartkopp et al. 1998). These shear forces are maximized when the hip and knee are placed in 90° of flexion (Hartkopp et al. 1998; McHenry and Shields 2012). We previously developed a low-force exercise using neuromuscular electrical stimulation to reduce this fracture risk. We used a train of muscle twitches that limits muscle force development and challenges the contractile and metabolic machinery of skeletal muscle (Petrie et al. 2015). Our low-force exercise increased the expression of early transcription factors needed for the transformation of skeletal muscle to a fatigue resistant phenotype. However, the training dose needed for a safe, feasible, and efficacious low-force exercise intervention for people with chronic paralysis was not known. In this study, we found that doses of low-force exercise training for at least 12 weeks resulted in larger improvements in fatigability. Similarly, frequent ~90-minute training sessions (>3 times per week) increased the expression of selected metabolic and contractile genes. To our knowledge, this is the first study to explore the dose-response of low-force exercise training in chronically paralyzed human skeletal muscle. These results are consistent with other exercise recommendations for people without a spinal cord injury (Murphy et

al. 2010; Nelson et al. 2007). However, unique to our exercise intervention is the use of paralyzed skeletal muscle that experiences little to no other activity during the day.

Exercising paralyzed skeletal muscle requires the use of an external stimulator because of an upper motor nerve lesion impairing communication between the brain and alpha-motor neuron. Neuromuscular electrical stimulation depolarizes peripheral alpha-motor neuron axons to evoke muscle contractions. Unique to these evoked contractions using neuromuscular electrical stimulation is the opportunity to recruit nearly all muscle fibers for a single muscle twitch. During volitional muscle contractions, muscle fibers are recruited only as needed to complete a movement or obtain the desired force output (Henneman 1957; Henneman et al. 1965). Therefore, only during very high-force contractions can nearly all muscle fibers be recruited. The novelty of our exercise is the associated low-force developed during a single twitch with nearly all muscle fibers recruited. Paralyzed human skeletal muscle acutely responds to a low-force exercise using neuromuscular electrical stimulation by increasing expression of early stress response genes like PGC1A, ABRA, and NR4A3 (Petrie et al. 2015). When dosed appropriately, our findings suggest that our low frequency exercise may change the gene expression signature of chronically paralyzed muscle to improve fatigability and metabolic capacity.

Few studies have linked the recently discovered mitochondrial pyruvate carrier 1 and 2 (MPC1 and MPC2, respectively) to exercise (Bricker et al. 2012a; Bricker et al. 2012b). MPC1 and MPC2 are mitochondrial transport proteins that bridge cytosolic glycolysis with the mitochondrial tricarboxylic acid cycle (TCA). The intra-membrane proteins MPC1 and MPC2 transport pyruvate into the mitochondrial from the cytosol to be oxidized. Because the TCA is the critical energy producer during exercise, MPC2 and

MPC1 are crucial for glucose metabolism. Exercise is a potent trigger to initiation of mitochondrial biogenesis through stimulation of early response genes like PGC-1α (Adams et al. 2011; Egan et al. 2010; Mootha et al. 2003). Therefore, exercise is a likely trigger for the expression of MPC1 and MPC2. We have previously shown that MPC1 and MPC2 expression is increased in human paralyzed muscle after years of high-force exercise training using neuromuscular electrical stimulation, but is not altered immediately after a bout of exercise (Petrie et al. 2015; Petrie et al. 2014b). Here, we found a positive association between the expression of MPC1 and MPC2 to changes in fatigue index. Further, we found that MPC1 and MPC2 expression was positively associated with PGC-1α and MYH2 expression, while negatively associated with ACTN3 expression. PGC-1α is often considered a master regulator of skeletal muscle gene expression important for the development of a fatigue-resistance, metabolically active phenotype. MYH2 is a myosin protein predominantly expressed in muscle fibers with a fatigue resistant phenotype, whereas ACTN3 is more closely associated with a fast-fatigable phenotype. The co-expression of these key genes suggests that with enough low-force exercise training, we may be able to improve the phenotype of chronically paralyzed human skeletal muscle.

We sought to define a safe and feasible dose of low-force exercise using neuromuscular electrical stimulation for people with chronic paralysis. We found the number of training sessions performed per week provided the best estimate for changes in physiologic biomarkers (fatigability and gene expression). Confounding our results is the interaction between the number of stimulus pulses, the number of training sessions per week, and number of weeks trained. The people that trained more frequently also trained with more stimulus pulses per session and for a longer training period. We had a wide range of training compliance, but few subjects trained with more than 3 sessions per week. This

small sample size limits our ability to determine definitively the effect of low-force exercise training on chronically paralyzed muscle. However, our goal was to establish an estimate for the effect size of low-force exercise training in people with chronic spinal cord injury. Based our results, we suggest future work use 90-minute training sessions performed at least 5 times per week for at least 12 weeks in order to maximize the benefits of a low-frequency stimulation protocol.

*Summary and Conclusions*

The significant musculoskeletal deterioration after a spinal cord injury necessitates the development of safe, feasible, and cost effective interventions to promote the musculoskeletal health in the paralyzed extremities. For the first time, we establish an estimate for the relationship between training duration and changes in physiologic biomarkers after low-force exercise training in people with paralysis. Interestingly, we found a strong relationship between the change in muscle fatigability and expression of MPC1 and MPC2, critical proteins for the transport pyruvate into the mitochondria. Additionally, we found strong associations between MPC1 and MPC2 expression with other genes related to metabolism, like PGC-1α. Whether low-force exercise training of appropriate dose can promote improve musculoskeletal health in people with spinal cord injury is unknown. Future work is needed to determine how appropriately dosed low-force exercise training influences the health of people with spinal cord injury.

## Table

**Table 5.1 Chapter 5 subject characteristics**

| Subject | Sex | Age | Injury Level | Years Post Injury |
|---|---|---|---|---|
| 01 (RS) | M | 27 | T8 | 7 |
| 02 (TB) | M | 62 | T12 | 4 |
| 03 (MW) | M | 33 | T4 | 11 |
| 04 (KS) | M | 26 | C4/5 | 2 |
| 05 (RJ) | M | 49 | T4 | 5 |
| 06 (AV) | M | 31 | T10 | 5 |
| 07 (JR) | M | 35 | T9 | 13 |

## Figures

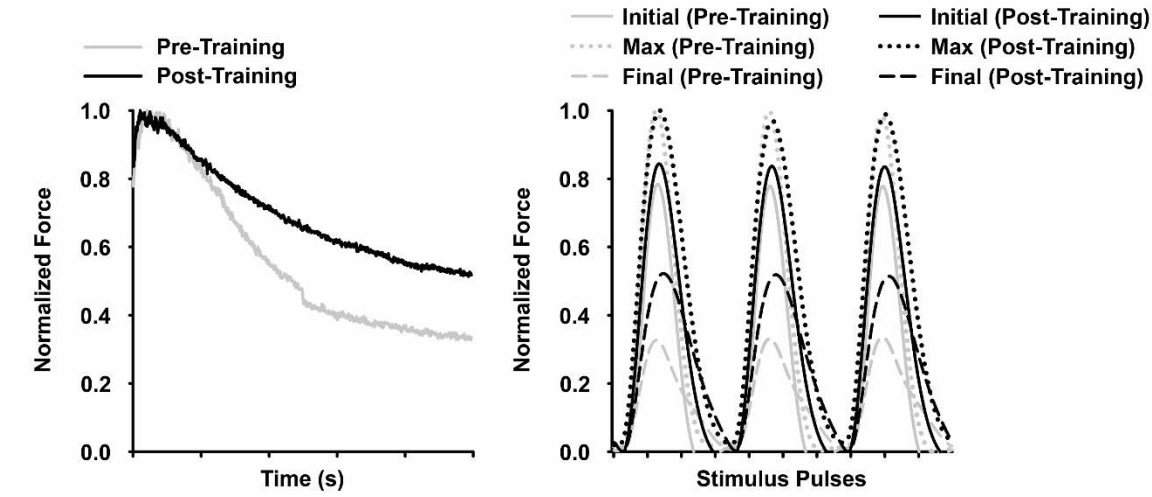


**Figure 5.1 Representative example of subject 5's muscle performance test**
Each stimulus pulse delivered to the muscle results in a twitch-like muscle contraction (Right). At the beginning of the train, the force developed during each stimulus pulse increases (potentiation) until reaching a maximum. After the maximum force is reached, the force developed during stimulus pulses decreases (fatigue). The pattern of force development across 1,000 stimulus pulses (bout 1) before and after muscle training is illustrated for subject 5 (Left).

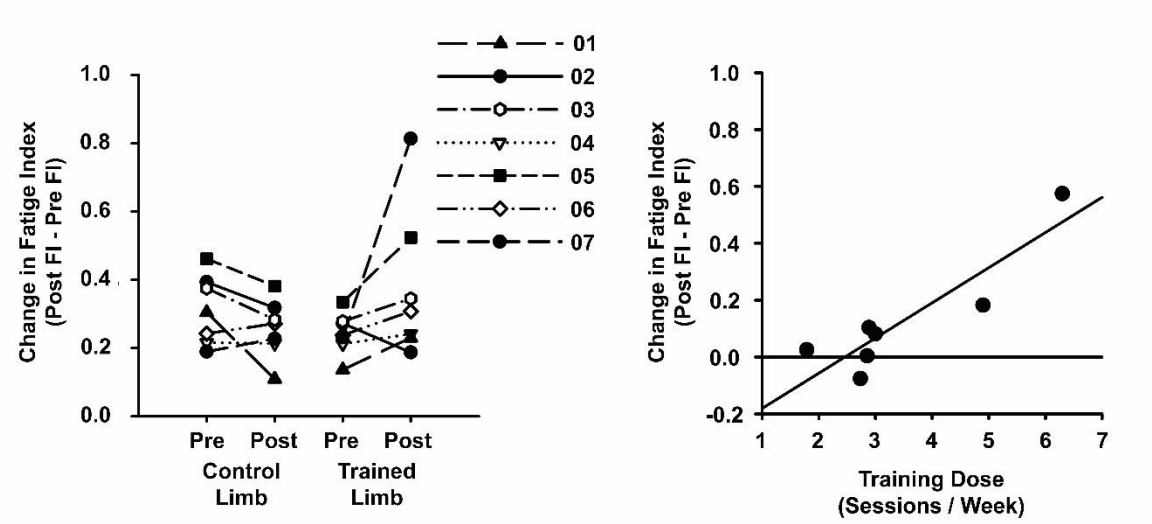


**Figure 5.2 Muscle fatigue before and after low-force exercise training**
Each subject's fatigue index from the muscle performance test before and after at least 12-weeks of training in the trained and control limb (Left). The relationship between the training dose as measured by the number of training sessions delivered compared to the change in fatigue index after at least 12-weeks of training using the low-force exercise protocol. There was a significant linear correlation between the training dose and change in fatigue index (p=0.006) (Right).

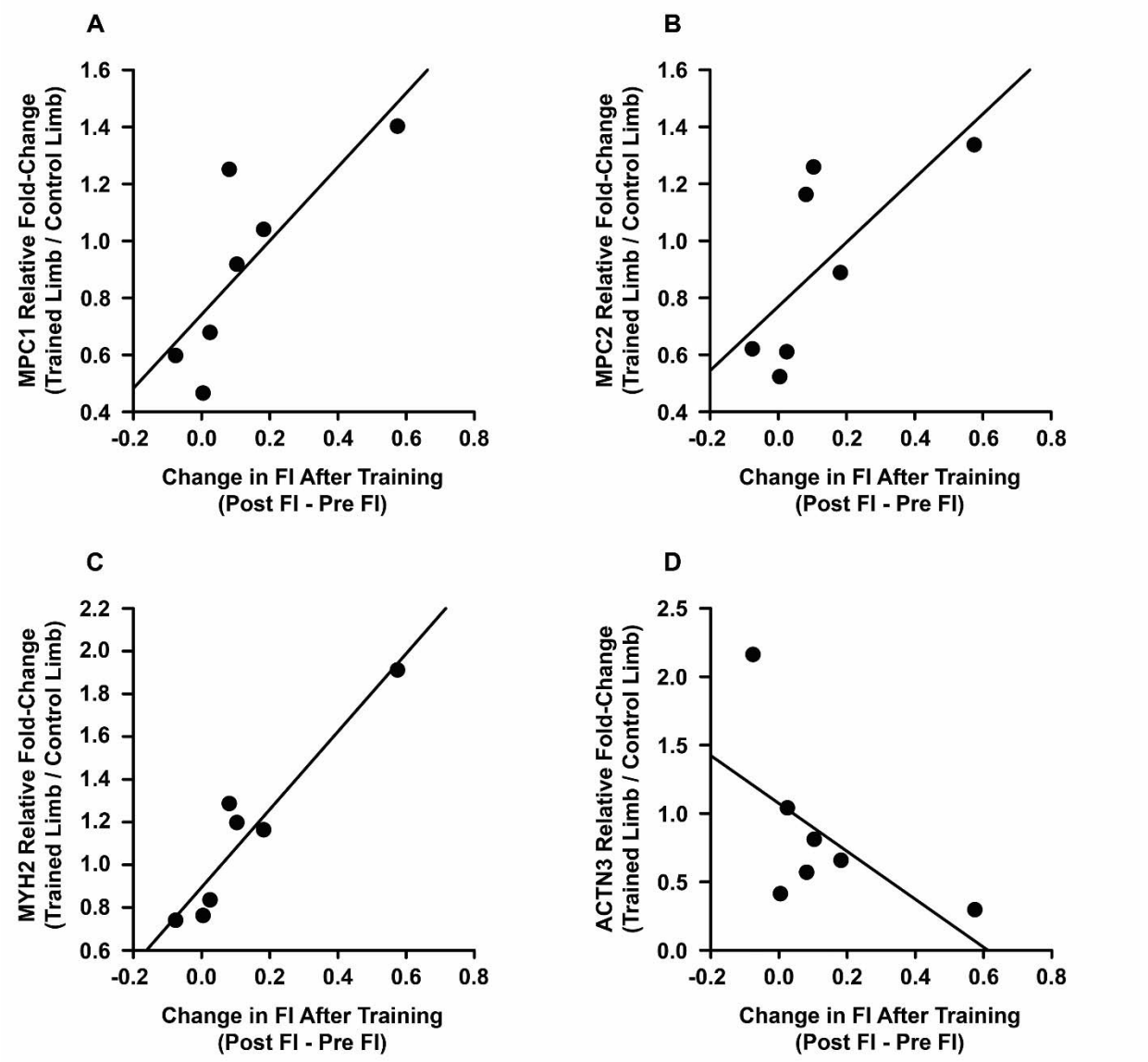


**Figure 5.3 Change in fatigue index consistent with gene expression**
There was a consistent relationship between the change in fatigue index after at least 12 weeks of training and the expression of key genes indicative of a more fatigue resistant phenotype. There was a positive linear association between the change in fatigue index and MPC1 expression (R2 = 0.63) (A). There was a positive linear association between the change in fatigue index and MPC2 expression ($R^2$ = 0.50) (B). There was a positive linear association between the change in fatigue index and MYH2 expression ($R^2$ = 0.89) (C). There was a negative linear association between the change in fatigue index and ACTN3 expression ($R^2$ = 0.35) (D).

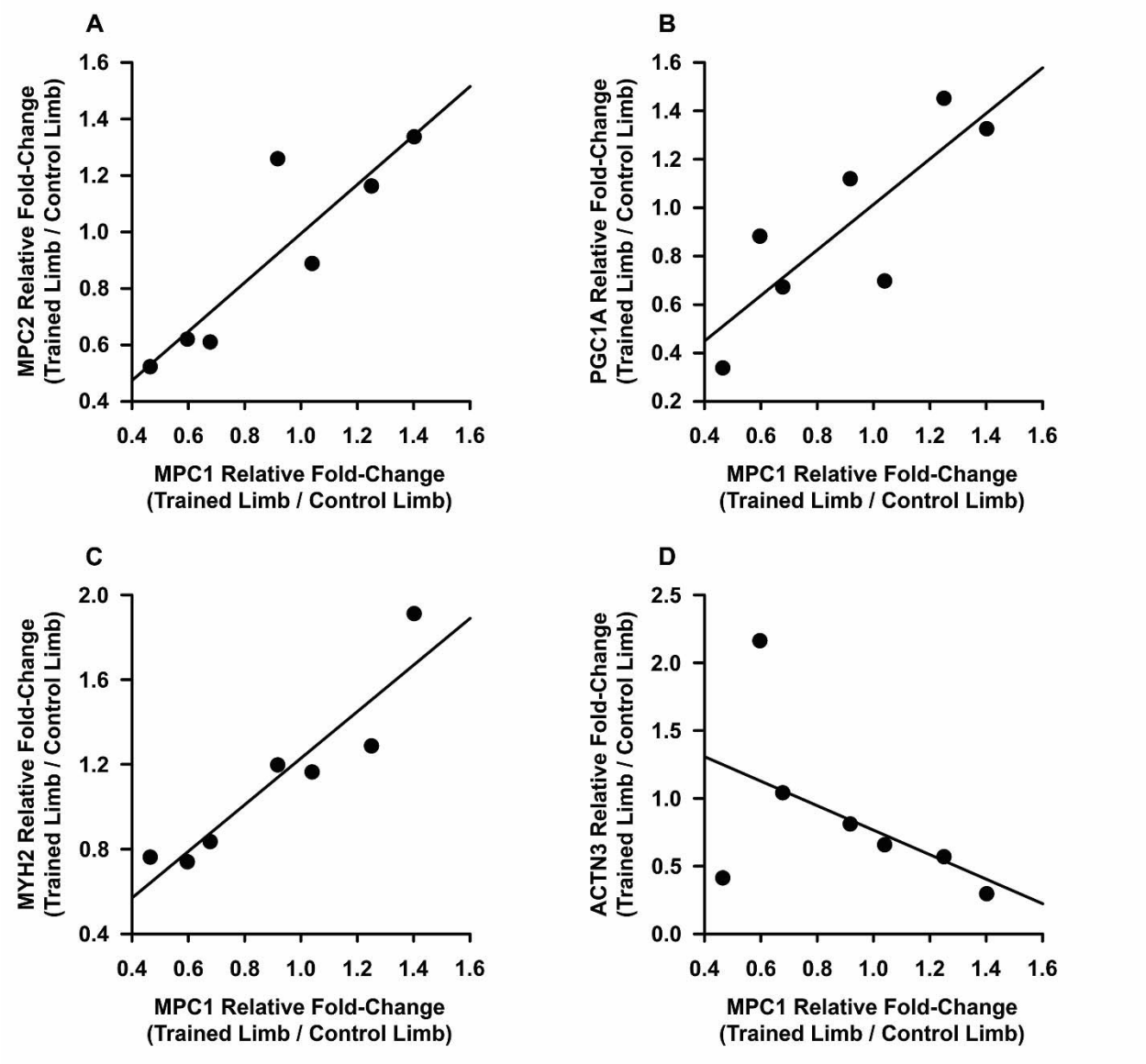


**Figure 5.4 Association of MPC1 expression to other key genes for a fatigue resistant phenotype**
There was a consistent relationship between the expression of important metabolic gene MPC1 to other key metabolic and contractile gene indicative of a more fatigue resistant phenotype. There was a positive linear association between the expression of MPC1 and MPC2 ($R^2$ = 0.79) (A). There was a positive linear association between the expression of MPC1 and PGC-1α ($R^2$ = 0.64) (B). There was a positive linear association between the expression of MPC1 and MYH2 ($R^2$ = 0.86) (C). There was a negative linear association between the expression of MPC1 and ACTN3 ($R^2$ = 0.25) (D).

# CHAPTER 6 CONCLUSIONS

Routine physical activity is important to maintain musculoskeletal and systemic health. The loss of routine physical activity leads to severe musculoskeletal deterioration and metabolic dysfunction. Rehabilitation interventions using high-force exercises using neuromuscular electrical stimulation help preserve the musculoskeletal system when started early after a spinal cord injury. Weakened musculoskeletal systems make these interventions unsafe for people with long-term paralysis. The purpose of this research was to 1) develop a gene expression signature after a single dose and long-term training using a high-force exercise in people with an acute spinal cord injury; 2) develop a novel low-force exercise intervention using neuromuscular electrical stimulation to limit force production and increase routine physical activity for chronically paralyzed human skeletal muscle; 3) determine the gene expression signature after a single dose of this novel low-force exercise in people with long-term paralysis; 4) develop a dose estimate of this low-force exercise needed to initiate a phenotype transformation of chronically paralyzed skeletal muscle.

## Specific Aim 1

*Hypothesis 1.1*

A single dose of high-force electrically stimulated muscle activity will increase the expression of metabolic transcription factors key for the transformation of muscle fibers from fast to slow.

*Supported:* There was over a 5-fold increase in the expression transcription factors for the transformation (PGC-1α, NR4A3, and ABRA) needed to transform skeletal muscle to a more fatigue resistant, slow-twitch phenotype.

*Hypothesis 1.2*

Long-term training with high-force electrically stimulated muscle activity will increase the expression of metabolic and contractile protein genes associated with slow, oxidative muscle fibers.

*Supported:* Long-term unilateral training (>4 years) using a high-force training exercise in people with paralysis increased the expression genes associated with the slow-twitch phenotype (PGC-1α, MYH7, MYL3, and MYH6) and decreased the expression of myostatin, a positive regulator of muscle atrophy.

## Specific Aim 2

*Hypothesis 2.1*

Low-force exercise using electrical muscle activity will cause more muscle fatigue in paralyzed human skeletal muscle compared to non-paralyzed human skeletal muscle.

*Supported:* Our novel low-force exercise using neuromuscular electrical stimulation delivered at a 3Hz frequency significantly fatigue chronically paralyzed muscle compared to non-paralyzed skeletal muscle.

*Hypothesis 2.2*

Low-force exercise using neuromuscular electrical stimulation will increase post-activation potentiation in chronically paralyzed human skeletal muscle compared to non-paralyzed human skeletal muscle.

*Supported:* Our novel low-force exercise induced significant post-activation potentiation in chronically paralyzed, but it was minimally induced in non-paralyzed human skeletal muscle.

*Hypothesis 2.3*

Chronically paralyzed human skeletal muscle will express genes consistent with a highly fatigable muscle fiber phenotype, while non-paralyzed human skeletal muscle will express genes consistent with a fatigue-resistant muscle fiber phenotype.

*Supported:* Chronically Paralyzed human skeletal muscle had a gene expression signature consistent with highly fatigable muscle fibers. Chronically paralyzed muscle had higher concentrations of MSTN, ANKRD1, and MYH8 consistent with high fatigable muscle fibers. In contrast, non-paralyzed muscle had higher concentrations of MYL3, SDHB, PDK2, and RYR1 consistent with the more fatigue resistant muscle fibers.

## Specific Aim 3

*Hypothesis 3.1*

A single dose of exercise using 5Hz neuromuscular electrical stimulation will fatigue chronically paralyzed human skeletal muscle more than a dose of exercise using 20Hz neuromuscular electrical stimulation.

*Not Supported:* A single bout of our novel low-force exercise using neuromuscular electrical stimulation delivered at 5Hz fatigued chronically paralyzed muscle to the same extent as a higher force exercise using neuromuscular electrical stimulation delivered at 20Hz.

*Hypothesis 3.2*

A single dose of exercise using 5Hz neuromuscular electrical stimulation will increase the expression of metabolic transcription factors more than a single dose of exercise using 20Hz neuromuscular electrical stimulation in chronically paralyzed human skeletal muscle.

*Not Supported:* A single bout of our novel low-force exercise using neuromuscular electrical stimulation delivered at 5Hz increased the expression of metabolic transcription factors (PGC-1α and NR4A3) to the same extent as a higher force exercise using neuromuscular electrical stimulation delivered at 20Hz.

*Hypothesis 3.3*

A single dose of exercise using 5Hz neuromuscular electrical stimulation will increase the gene expression of slow-twitch transcription factors more than a single dose of exercise using 20Hz neuromuscular electrical stimulation in chronically paralyzed human skeletal muscle.

*Not Supported:* A single bout of our novel low-force exercise using neuromuscular electrical stimulation delivered at 5Hz changed the expression of slow-twitch transcription factors and contractile proteins (ABRA and MSTN) to the same extent as a higher force exercise using neuromuscular electrical stimulation delivered at 20Hz.

## Specific Aim 4

*Hypothesis 4.1*

There will be a strong positive correlation between the training dose (number of training sessions per week) of a low-force exercise using neuromuscular electrical stimulation and change in fatigability of chronically paralyzed human skeletal muscle.

*Supported:* We found a strong positive association between the number of training sessions performed weekly and the change in muscle fatigability after at least 12 weeks of training using the low-force exercise.

*Hypothesis 4.2*

There will be a strong positive correlation between the training dose (number of training sessions per week) of a low-force exercise using neuromuscular electrical stimulation and the change in expression of key genes indicative of a fatigue-resistant phenotype transformation in chronically paralyzed human skeletal muscle.

*Supported:* Consistent with the changes in fatigability, we found a strong positive association between the number of training sessions performed weekly and expression of MPC1, MPC2, and MYH2 after at least 12 weeks of training using the low-force exercise.

*Hypothesis 4.3*

There will be a strong positive correlation between the change in fatigability and the expression of genes indicative of a transformation to a more fatigue resistant phenotype after low-force exercising training using neuromuscular electrical stimulation in people with chronic spinal cord injury.

*Supported:* We found a strong positive correlation between the change in fatigue index after low-force exercise training and the expression of MPC1, MPC2, and MYH2 after at least 12 weeks of training.

## Summary

There is a need to help improve the health and wellness of people with spinal cord injury. Exercise is a powerful physiologic stressor for skeletal muscle. People with spinal

cord injury are unable to exercise their paralyzed limbs volitionally. Interventions like neuromuscular electrical stimulation provide a novel alternative to exercise paralyzed limbs that would otherwise be inactive. Traditional uses of neuromuscular electrical stimulation result in high-force contractions that can put bone at risk for fracture, particularly for people with long-term spinal cord injury. There is a need to develop an alternative intervention to exercise paralyze muscle at low levels of force to reduce the risk of injury to underlying bone. The goal of this research was to develop a novel exercise intervention that could safely and feasibly exercise chronically paralyzed muscle without loading the underlying osteoporotic bone. We found trains of low frequency stimulation induced muscle fatigue and post-activation in chronically paralyzed muscle, increased the expression of metabolic transcription factors, and decreased the expression of regulators of muscle atrophy. We probed into the dose of our low-force exercise needed to start transforming the phenotype of chronically paralyzed muscle. We suggest at least 5 days of training be performed weekly during future studies to help maximize the change physiologic biomarkers, such as fatigue and expression of metabolic genes (MPC1 and MPC2). Together, this work demonstrates the unique ability for a low-force exercise using neuromuscular electrical stimulation to challenge chronically paralyzed human skeletal muscle. How this low-force exercise influences the systemic health of people with spinal cord injury remains unknown. Unilateral muscle training, as performed here, is unlikely change systemic metabolic biomarkers because of the limited muscle mass participating in the exercise. Future studies will determine if long-term training using a low-force exercise is a feasible and efficacious intervention to improve the metabolic capacity of skeletal muscle, restore skeletal muscle's ability as a primary blood glucose regulator, and improve the health and quality of life of people with chronic spinal cord injury.

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