Characterization of Histological Changes in the Microvasculature of Rat Skeletal Muscle After Spinal Cord Injury

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CHARACTERIZATION OF HISTOLOGICAL CHANGES IN THE MICROVASCULATURE OF RAT SKELETAL MUSCLE AFTER SPINAL CORD INJURY

by

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CHARACTERIZATION OF HISTOLOGICAL CHANGES IN THE MICROVASCULATURE OF RAT SKELETAL MUSCLE AFTER SPINAL CORD INJURY

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The purpose of this study was to determine whether there are histological changes in the microvasculature of rat skeletal muscle following chronic spinal cord injury both above and below the level of injury. This study is important because microvascular structure likely impacts muscle performance and cardiovascular health. To the best of our knowledge, this is the only study to investigate microvascular structure within rat skeletal muscle after spinal cord injury. We hypothesized structural remodeling would occur in both the myofibers and microvasculature, which would then manifest in differences in myofiber cross sectional area and microvascular diameter, wall thickness, wall to lumen ratio, and wall cross sectional area.

Changes in sympathetic tone and reduced muscular activity following spinal cord injury may induce microvascular structural remodeling. Initially after injury, sympathetic activity below the level of injury is diminished. Over time, neuroplasticity results in recovery of sympathetic tone, which increases vascular smooth muscle contraction and may lead to alterations in vasculature structure. In addition, the spinal lesion leads to loss of descending drive, which causes physical deconditioning below the level of injury. Physical deconditioning is known to induce vascular remodeling, and effects may be opposite of those associated with increased sympathetic tone.

We conducted a test of vascular remodeling in a rat contusion model of spinal cord injury. Ten adult female rats were evenly divided into control and spinal cord injury groups. Severe spinal cord injury was induced using a controlled weight drop onto the spinal cord, resulting in a contusion injury. After a 90 day survival period, the biceps brachii, triceps brachii, tibialis cranialis, and soleus muscles were removed, processed, and stained with Verhoeff van Gieson elastin and hematoxylin and eosin stains for histological analysis. Ultrastructural features of the myofibers and non-capillary microvessels were quantified. There was no significant difference between spinal cord injury and control skeletal muscles with regards to muscle cross sectional area, myofiber cross sectional area, microvascular diameter, wall thickness, wall to lumen ratio, or wall cross sectional area. Results indicated similar myofiber integrity and microvascular structure between control and spinal cord injury groups above and below level of injury.

While results did not support our original hypothesis, the findings also did not contradict previous studies. Following chronic spinal cord injury, recovery of spontaneous muscle activation and sympathetic activity may maintain integrity of skeletal muscle and associated microvasculature. Future research could assess microvascular function post spinal cord injury and identify an alternate animal model to study effects of spinal cord injury on muscle atrophy and associated microvasculature changes.
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SCI: spinal cord injury
AD: autonomic dysreflexia
BBB: Basso, Beattie, and Bresnahan
PBS: phosphate-buffered saline
CTL: control
BB: biceps brachii
TB: triceps brachii
TC: tibialis cranialis
S: soleus
CHAPTER 1: BACKGROUND AND INTRODUCTION

1.1 Thesis Motivation and Introduction

The purpose of this study was to determine whether there are histological changes in the microvasculature of rat skeletal muscle following chronic spinal cord injury (SCI), which is important because histological changes are likely to impact muscle performance and overall cardiovascular health. Adequate blood circulation is critical for delivering needed substrates and transporting metabolic waste byproducts from muscle fibers in order to prevent muscle fatigue. Because flow resistance in microvessels is the primary determinant of systematic vascular resistance, structural microvascular adaptations are likely to impact overall cardiovascular health by altering vascular resistance. Changes in sympathetic tone and muscle atrophy likely induce microvascular remodeling. Results from this study will increase understanding of how SCI impacts peripheral microvasculature and may help explain why there is a higher incidence of cardiovascular disease in individuals with SCI compared to able-bodied individuals (Myers, Lee, & Kiratli, 2007).

Disruptions in the autonomic nervous system following SCI cause changes in sympathetic tone, which may induce vascular remodeling. Initially after injury, there is a period of “spinal shock” manifesting as diminished sympathetic activity below the level of injury and reduced muscular tone in the motor system (Teasell, Arnold, Krassioukov, & Delaney, 2000). Descending spinal pathways to the cardiovascular system are altered, resulting in decreased sympathetic activity and unopposed parasympathetic tone via the intact vagal nerve (Claydon, Steeves, & Krassioukov, 2006). Reduced sympathetic tone and loss of brainstem control result in
various cardiovascular dysfunctions, including hypotonia, areflexia, and postural hypotension (Atkinson & Atkinson, 1996; Calaresu & Yardley, 1988; Cragg, Noonan, Krassioukov, & Borisoff, 2013; Gondim et al., 2004). Over time, the nervous system responds to the injury by establishing new synaptic connections and altering strength of existing synapses in a process known as neuroplasticity. Sympathetic tone gradually recovers to exceed able-bodied baseline levels (Brown & Weaver, 2012; A. V. Krassioukov & Weaver, 1996a). Neural remodeling is linked to chronic complications of high-level spinal cord injury, such as autonomic dysreflexia (Brown & Weaver, 2012; Gunduz & Binak, 2012; Popa et al., 2010). These changes in sympathetic tone are likely to induce vascular remodeling, because blood vessels are dynamic and respond to changes in the hemodynamic environment.

A spinal lesion leads to muscle atrophy and to vascular deconditioning due to physical inactivity below the level of injury (West, Alyahya, Laher, & Krassioukov, 2013). As a result, distal musculature below the level of injury exhibits significant atrophy and deconditioning characterized by reduced potential for oxidative metabolism, reduced fiber cross sectional area, and a shift towards the fast fiber type (Castro, Apple, Staron, Campos, & Dudley, 1999; Shields, 2002; Stewart et al., 2004). These changes culminate in greater muscle fatigability and induce changes in the vascular smooth muscle. The effect of physical inactivity on arterial structure has been explored via various models, including hindlimb unloading and bed rest. Despite some inconsistencies, overall results indicate occurrence of vascular remodeling, including reduced diameter, reduced wall caliber, and impaired endothelial function (Trappe et al., 2009; Tyml, Mathieu-Costello, Cheng, & Noble, 1999; Tyml & Mathieu-Costello, 2001).

Surprisingly, morphological changes in peripheral microvasculature as a result of SCI have not been investigated (to the best of our knowledge). Most studies investigating
cardiovascular effects following SCI have focused on central cardiovascular control (West et al., 2013). However, due to the importance of skeletal muscle microvasculature to muscle performance and cardiovascular health, morphological adaptations within peripheral microvessels are likely to be of high clinical significance.

We hypothesized that vascular atrophy occurs within skeletal muscle microvessels following SCI. In order to test this hypothesis, we conducted a histological study. Ten female rats were evenly divided into control and SCI groups, and a contusion injury was used to induce SCI. After a 90 day survival period, a set of postural and phasic skeletal muscles from both the forelimb and hindlimb was removed, processed, and stained with the Verhoeff van Gieson elastin stain. Histological analysis is commonly used to study the microscopic anatomy of muscles and blood vessels. Fibers and non-capillary microvessels within the muscle cross sections were characterized. The control and SCI groups were compared using statistical testing.

1.2 SPINAL CORD INJURY

1.2.1 Overview and Epidemiology

Spinal cord injury is a devastating medical condition with severe functional, psychological, and socio-economic consequences. Most individuals with SCI experience some extent of paralysis ranging from incomplete paraplegia to complete tetraplegia, and less than 1% of all occurrences result in complete neurologic recovery by hospital discharge (University of Alabama at Birmingham, February 2015). In addition to severe motor and sensory dysfunction, chronic SCI is also associated with numerous other complications, including cardiovascular complications, respiratory failure, bladder dysfunction, neurogenic bowel, spasticity, and pain (Furlan & Fehlings, 2008; Garshick et al., 2005). Because of these complications, SCI is often
life-altering, requires large lifestyle changes, and may lead to psychological despair and mental anguish.

SCI has significant socio-economic impact, and individuals with SCI involuntarily place an enormous burden on the healthcare system. Estimated first-year treatment cost ranges from $218,504 for individuals with incomplete motor function at any level to $741,425 for high complete tetraplegia injuries (in May 2006 dollars). Ongoing direct medical costs for individuals with chronic SCI are estimated to be approximately $21,450 annually per person, along with additional costs from complications (in fiscal year 2005 costs) (French et al., 2007). Estimated lifetime cost of chronic SCI ranges from $1.5 million for incomplete paraplegia to $3.0 million for complete tetraplegia (Krueger, Noonan, Trenaman, Joshi, & Rivers, 2013). Within the United States, total SCI-related costs are estimated at $9.7 billion annually (Berkowitz, O’Leary, Kruse, & Harvey, 1998), and the number of individuals living with SCI in 2014 is estimated to be approximately 276,000 persons with a range of 240,000 to 337,000 persons (University of Alabama at Birmingham, February 2015). In addition, there are indirect costs, such as loss of productivity and decreased contribution to society.

Occurrence of complications depends on level of injury and time after injury. Injuries occurring higher on the spinal column impact more spinal pathways and result in more complications compared to those occurring at a lower level. In the acute phase, the primary injury results in neurological damage within the spinal cord due to mechanical injury. The primary injury catalyzes a series of biological events, referred to as secondary injury. Secondary injury occurs over the time course of minutes to weeks and is characterized by an inflammatory response to primary injury, which exacerbates injury by causing additional neurological damage. Additional neurological impairments occur in the chronic phase spanning the time course of days
to years after injury and include myelin degradation and axonal disruption (Silva, Sousa, Reis, & Salgado, 2014).

Over the last decade, there have been major changes in morbidity and mortality among the SCI population. Historically, renal and pulmonary diseases have been leading causes of mortality among individuals with SCI (Devivo, 2012). Due to medical advancements in providing acute care and treating renal and pulmonary complications, cardiovascular disease has emerged to become the leading cause of mortality in the SCI population (Cragg et al., 2013; Cragg et al., 2013; Gondim et al., 2004).

There is a higher prevalence of cardiovascular disease among individuals with SCI compared to the able-bodied population (Krum et al., 1992; Myers et al., 2007; Phillips et al., 1998; West et al., 2013) that is not fully explained by traditional risk factors, such as physical inactivity, cholesterol level, resting blood pressure (BP), glucose intolerance, and smoking status (Krum et al., 1992; Phillips et al., 1998; West et al., 2013). For instance, high thoracic or cervical SCI typically results in autonomic disturbances that cause low resting blood pressure, which is usually considered cardio-protective. However, individuals with high thoracic or cervical SCI exhibit a larger prevalence of cardiovascular disease compared to the able-bodied population (West et al., 2013).

These observations suggest that there may be other effects of SCI not currently studied that contribute to the elevated risk of cardiovascular disease, irrespective of traditional risk factors. Given these implications, additional research to increase fundamental understanding of how the cardiovascular system changes after SCI is needed.
1.2.2 Disruptions in the Autonomic Nervous System

The autonomic nervous system is a part of the peripheral nervous system that regulates key involuntary functions within the body. As such, it is responsible for proper cardiovascular control, controls involuntary responses, and relays information from the central nervous system to target organs. The autonomic nervous system comprises two antagonist divisions that collectively provide balanced autonomic control and are activated in accordance to demand. The sympathetic nervous system is considered excitatory and responsible for the body’s fight or flight response, while the parasympathetic nervous system is considered suppressive and dominates at rest. Unlike most organs that receive innervation from both divisions of the autonomic nervous system, most blood vessels only receive sympathetic innervation. The cerebral and pulmonary blood vessels are exceptions that also receive parasympathetic innervation (Suzuki & Hardebo, 1993).

The sympathetic nervous system and parasympathetic nervous system share some organizational similarities despite functional differences. Both systems have two neuronal populations, the preganglionic and postganglionic neurons. Preganglionic neurons have cell bodies located in the gray matter of the brain and spinal cord, travel in the anterior roots of the spinal cord and cranial nerves, and form synapses on postganglionic neurons. Postganglionic neurons are located in the autonomic ganglia of the peripheral nervous system and form synapses with target organs. Both sympathetic and parasympathetic preganglionic neurons are cholinergic (West et al., 2013).

Peripheral vasculature only receives innervation from the sympathetic nervous system. Sympathetic preganglionic neurons originate in the thoracic spinal cord gray matter (T1 – T12) and the upper lumbar segments of the spine (L1 – L2). Sympathetic preganglionic axons exit
through anterior roots of the spinal cord and form synapses with postganglionic sympathetic neurons within the sympathetic chain ganglia and prevertebral ganglia. Sympathetic postganglionic neurons are mostly adrenergic and release norepinephrine. The sympathetic innervation most important to cardiovascular control is that of the heart at T1 – T4, upper limb blood vessels at T1 – T4, and splanchnic bed and lower limb blood vessels at T6 – L2 (West et al., 2013).

The sympathetic nervous system innervates blood vessels throughout the body causing vasoconstriction to control local blood flow and vascular resistance. The central nervous system sends specific control signals to target tissues and organs in accordance with functional needs by means of reflexes and patterned regulation (McLachlan, 2007a). Sympathetic outflow arises in the thoracolumbar cord and is directly damaged by SCI involving regions or ventral roots between T1 and L3. In addition, injuries at the cervical level affect the descending pathways, thereby disconnecting sympathetic outflow from higher control centers. Preganglionic neurons in the upper thoracic segments innervate blood vessels in the head and neck, while neurons in the low thoracic and upper lumbar segments control those in the pelvic organs and lower limbs (McLachlan, 2007a).

SCI disrupts supraspinal control of spinal sympathetic circuits that ultimately innervate the adventitial and medial layers of the blood vessels, as sympathetic nervous system efferent activity below the level of injury is reduced (Claus-Walker & Halstead, 1982). Function in the spinal cord below the level of injury becomes independent from supraspinal control in a process termed “decentralization” of the sympathetic nervous system, which then leads to a multitude of vascular abnormalities (Claus-Walker & Halstead, 1982; Suzuki & Hardebo, 1993). Vascular dysfunction below the level of injury is characterized by decreased conduit artery diameter,
reduced blood flow, increased shear rate, and adrenoceptor hyper-responsiveness (Amiya, Watanabe, & Komuro, 2014). The changes in peripheral sympathetic tone are likely to cause vascular adaptations in the microvessels; given the importance of peripheral vasculature to cardiovascular health, any structural adaptations are likely to be of high clinical impact.

1.2.3 Changes in Sympathetic Tone

Sympathetic tone, which refers to the level of baseline sympathetic innervation, following SCI depends on level and severity of injury, and time after injury. The higher and more severe the injury, the greater the change in sympathetic tone will be. Initially after injury, there is a period of “spinal shock” manifesting as diminished sympathetic activity below the level of injury in the sympathetic nervous system and reduced muscular tone in the motor system (Teasell et al., 2000). Although peripheral tone is preserved and intact, it is not adequate to maintain arterial blood pressure following high thoracic or cervical SCI (Shields, 2002). In the first few weeks, lack of sympathetic control of the vasculature and unopposed parasympathetic activity via the intact vagal nerve result in hypotonia, areflexia, and postural hypotension as there is loss of important brainstem control of the vasculature (Amiya et al., 2014; Atkinson & Atkinson, 1996; Calaresu & Yardley, 1988; Cragg et al., 2013). Sympathetic tone gradually recovers over time to exceed able-bodied baseline.

Neuroplasticity and remodeling following SCI drive much of the change in sympathetic tone over time. Neuroplasticity encompasses a variety of responses within the injured cord, brain, and ganglia outside the central nervous system and ranges from structural changes to changes in membrane ion channels (Brown & Weaver, 2012). Disruption of descending pathways to the sympathetic preganglionic neurons results in a partial deafferentation. Intrinsic and dorsal root afferent inputs are left intact, and remaining local circuits are capable of
reorganization (A. V. Krassioukov & Weaver, 1996b; A. V. Krassioukov, Bunge, Pucket, & Bygrave, 1999). Although spinal sympathetic preganglionic neurons exhibit atrophy in the acute stage, normal morphology is regained within one month (A. V. Krassioukov & Weaver, 1996c; Teasell et al., 2000). Surviving terminals in ganglia generate and rapidly re-establish strong connections to preganglionic neurons or axons, perhaps even incorrectly to some postganglionic neurons (McLachlan, 2007a). Peripheral alpha-adrenoceptors in sympathetically denervated vascular beds may become hyper-responsive due to up-regulation in response to reduced sympathetic nervous system efferent output or decreased presynaptic noradrenaline re-uptake (McLachlan, 2007a). The remodeling in sympathetic circuits over time may underlie many cardiovascular abnormalities, including autonomic dysreflexia, following SCI (Brown & Weaver, 2012; A. V. Krassioukov & Weaver, 1996b).

1.2.4 Cardiovascular Complications

Occurrence of cardiovascular complications following SCI depends on both level of injury and time after injury. Generally, effects of sympathetic nervous system dysfunction below the level of injury are more severe with higher level injuries. Because of neuroplasticity and remodeling within the autonomic nervous system after injury, acute cardiovascular complications (up to 30 days following injury) differ from chronic cardiovascular complications (from 30 days to years after injury).

Acute cardiovascular complications following high-thoracic or cervical SCI are driven by neurogenic shock, which is characterized by the simultaneous presence of bradycardia and orthostatic hypotension. Bradycardia is defined as an abnormally slow heart rate that is usually lower than 60 beats per minute, and arterial hypotension is defined a systolic blood pressure below 90 mmHg and diastolic blood pressure below 60 mmHg (Popa et al., 2010). Orthostatic
hypotension is generally described as a sudden drop in systolic blood pressure of 20 mmHg or more or a decrease in diastolic blood pressure of 10 mmHg upon changing from a supine body position to an upright body posture (A. Krassioukov, Eng, Warburton, Teasell, & The SCIRE Research Team, 2009). Orthostatic hypotension results from loss of nervous system control of blood pressure and is exacerbated by loss of muscle tone that normally aids venous return of blood. Other important complications include increased vasovagal reflexes, venous stasis, and hypoxia (Atkinson & Atkinson, 1996; Cragg et al., 2013; Furlan & Fehlings, 2008; Popa et al., 2010). Risk of developing deep vein thrombosis increases, peaks 7 to 10 days after injury and during early phase of recovery and rehabilitation, and diminishes 8 to 12 weeks post injury (Popa et al., 2010). These cardiovascular complications are a result of the initial absence of sympathetic tone and unopposed vagal tone following a high-thoracic or cervical SCI.

Chronic cardiovascular complications following high-thoracic or cervical SCI are linked to neural remodeling (Brown & Weaver, 2012) and include impaired regulation of blood pressure, blood volume, and body temperature, autonomic dysreflexia, coronary heart disease and systemic atherosclerosis (Hagen, Rekand, Gronning, & Faerestrand, 2012; Popa et al., 2010).

1.2.5 Autonomic Dysreflexia

Autonomic dysreflexia is a life-threatening secondary condition of chronic SCI resulting from exaggerated spinal reflex sympathetic responses to peripheral stimuli originating below the level of injury (Gunduz & Binak, 2012). It generally occurs in individuals with SCI at T5 to T6 or higher and is characterized by a sudden bout of hypertension accompanied by bradycardia. Injury level, completeness of injury, and number of stimuli all contribute to severity and onset of autonomic dysreflexia (Gunduz & Binak, 2012). The most common stimulus is bladder distension resulting from urine retention or catheter blockage, and there are many other triggers,
such as pain or irritation, constipation, hemorrhoids, ingrown toenails, and pressure sores (Gunduz & Binak, 2012; Popa et al., 2010).

Afferent nerve impulses from the stimuli enter the spinal cord via the dorsal horn and ascend in the dorsal column and spinothalamic tract to activate the intermediolateral column of the spinal cord up to the level of injury (Gunduz & Binak, 2012; Karlsson, Friberg, Lonnroth, Sullivan, & Elam, 1998a; Teasell et al., 2000). Activation of the decentralized sympathetic nervous system also generates a marked noradrenaline spillover below the level of injury, which activates the sympathetic nervous system to increase efferent activity (Karlsson et al., 1998a).

The increased sympathetic drive results in widespread vasoconstriction below the level of injury to affect vasculature within skeletal muscle, organs, and skin. Vasoconstriction of the splanchnic vasculature is most significant and causes increased vascular resistance and shunting of blood, thereby forcing congested blood to enter the general circulation (Gunduz & Binak, 2012). Collectively, increased resistance and higher fluid load generate a potentially catastrophic increase in blood pressure. During episodes of autonomic dysreflexia, systolic and diastolic blood pressures can increase to as high as 300mmHg and 200mmHg, respectively (Karlsson et al., 1998a).

Typically, a change in blood pressure activates various control mechanisms, including baroreceptors that stimulate the parasympathetic nervous system to lower blood pressure. However, these mechanisms are disrupted after complete high thoracic or cervical SCI, so blood pressure is no longer properly regulated. The dysregulation is associated with an increase in calcitonin gene-related peptide-containing primary afferent arbors in the dorsal horn as part of remodeling post-injury (Krenz & Weaver, 1998). There is loss of descending inhibitory influences on spinal sympathetic reflexes (Gris, Marsh, Dekaban, & Weaver, 2005). During an
episode of autonomic dysreflexia, vasoconstriction is constrained to below the level of injury, and there is a lack of sympathetic tone above the level of injury due to activation of the parasympathetic nervous system in response to increased blood pressure (Brown & Weaver, 2012).

1.2.6 Physical Deconditioning

Individuals with SCI experience paralysis that may be alleviated to some extent through musculoskeletal and neuronal adaptations associated with rehabilitation. Initially, muscular tone is reduced, followed by increased skeletal muscle tone and spasticity over time in one or both legs (Dietz, 2012). Rehabilitation improves motor function in the affected limb through neuroplasticity of the central nervous system at various levels, including the spinal cord, brainstem, and cortex (Bruehlmeier et al., 1998; Dietz, 2012; Ding, Kastin, & Pan, 2005). This neuroplasticity compensates for some loss in function, as loss of function is mainly due to impaired supraspinal control over the neural circuitry in the spinal cord. The spinal neural centers underlying locomotion remain mostly intact, and neuronal circuits below the level of injury can be activated by afferent input (Dietz, 2012; Ding et al., 2005).

Skeletal muscle is primarily classified as either postural, phasic, or a mixture of the two. Postural muscles are composed of mostly slow-twitch fibers, predominantly used to maintain posture in the gravitational field, and prone to hyperactivity, meaning that they are less likely to experience disuse. Slow-twitch fibers have longer contraction duration, are better able to sustain work, and rely on aerobic respiration. Phasic muscles are composed of mostly fast-twitch fibers, primarily responsible for movement, and prone to disuse. Fast-twitch fibers are characterized by fast muscle contractions of short durations and rely on glycolysis (Trappe et al., 2009).
After SCI, there are significant changes in the morphology and contractility of muscles below the level of injury. Beginning 4 to 7 months post injury, there is a shift from normal type I slow and type II fast fibers to predominantly type II fast glycolytic fibers (Biering-Sorensen, Kristensen, Kjaer, & Biering-Sorensen, 2009; Burnham et al., 1997). The proportion of slow myosin heavy chain isomers progressively decreases while the proportion of hybrid fibers co-expressing both slow and fast myosin heavy chain isomers steadily increases (Shields, 2002). The increase in fast myosin isomers results in faster muscle contractile speed and physiological properties consistent with fast motor units - these changes result in higher muscle fatigability (Shields, 2002).

1.3 VASCULATURE

1.3.1 Overview

Proper blood flow is important for many bodily functions, including delivery of nutrients and oxygen to cells, waste transportation from cells, and maintenance of homeostasis. As the heart pumps, blood enters the aorta and is pushed through large conduit arteries into arteries of decreasing size, arterioles, and capillaries. Nutrient and gas exchange and waste diffusion occur within the thin-walled capillaries. Then, blood travels back to the heart via venules of increasing size and ultimately, veins.

Arteries and arterioles are made up of three main layers – the tunica intima, tunica media, and tunica adventitia. The lumen is the hollow internal cavity in which blood travels. Tunica intima, the innermost layer of the vascular wall, is a monolayer of endothelial cells connected to the arterial wall by a thin layer of connective tissue. Endothelial cells respond to signals and alterations in blood flow by catalyzing specific pathways for regulation of blood vessel lumen diameter. In large and medium-sized arteries, a layer of elastic tissue referred to as the internal
elastic lamina forms the outermost part of the tunica intima and separates the tunica intima from tunica media. Tunica media is the middle layer, comprises smooth muscle cells concentrically arranged around the long axis of the blood vessel intermingled with elastic fibers, and is primarily used for regulation of lumen diameter. The number of smooth muscle cell layers depends on vessel size with the number of layers varying from six within arteries of 300μm in diameter to one in the smallest arterioles (Asmar, Sassano, Demolis, Menard, & Safar, 1991; Gondim et al., 2004). Tunica adventitia is the outermost layer, comprised of irregularly-shaped collagen fibers, and used for scaffolding support to surrounding tissue.

Although arteries and arterioles have the same overall structure, each vessel has a specific structure reflecting its primary function. Elastic arteries are close to the heart, absorb much of the pressure directly from the heart, and contain more elastin. Large conduit arteries generate the Windkessel effect of providing constant pressure despite pulsatile blood flow and contain more smooth muscle cells, along with increased collagen and elastin (Kamisaka et al., 2008). Arterioles decrease the speed of blood flow to allow adequate time for nutrient and gas exchange within capillaries. Consequently, arterioles are the primary site of vascular resistance and contain just a few smooth muscle layers.

Smooth muscle cells exhibit a state of partial contraction at rest that is termed myogenic tone, which is the main source of vascular resistance and is very impactful to overall cardiovascular health. Both intrinsic and extrinsic factors regulate myogenic tone. Intrinsic factors include the myogenic response evoked by changes in intravascular pressure, endothelial factors, local chemical substances and hormones, metabolic by-products, and tissue hypoxia. While the specific compounds involved are unclear, it is generally accepted that intrinsic myogenic tone regulation is a property of smooth muscle cells within the medial layer involving
membrane potential depolarization, calcium influx through the L-type voltage-gated calcium channels, and change in actin and myosin regulatory enzymes to promote actin myosin-based contraction (Roman & Van Dokkum, 2014; Somlyo & Somlyo, 1994). Extrinsic factors include neural and hormonal factors. Sympathetic nerve fibers innervate all arteriolar smooth muscle cells and release norepinephrine that acts via α-adrenoceptors to cause vasoconstriction. Other substances primarily responsible for determining arteriolar diameter include nitric oxide, endothelin, vasopressin, and angiotensin (West et al., 2013).

### 1.3.2 Microvasculature of Skeletal Muscle

Blood flow to skeletal muscle is supplied by primary arteries that divide into feed arteries which then branch into a vast network of arterioles and capillaries. Primary arteries are positioned strategically along the long axis of the muscle and control the total amount of blood entering the muscle (Korthuis, 2011). Primary arteries represent the last branches of the arterial supply prior to entry into tissue and give rise to feed arteries that travel towards the epimysium of the muscle at right or oblique angles to the primary arteries. Feed arteries account for approximately 30-50% of total resistance to blood flow through the muscle, regulate blood flow proximal to microvessels embedded in muscle tissue, and branch into the arteriolar network upon muscle entry (Lash & Bohlen, 1992). Arterioles enter the perimysium and travel perpendicular to the muscle fiber axis until branching into smaller arterioles. Terminal arterioles are the last arteriolar branch to contain vascular smooth muscle cells and represent the smallest functional unit for blood flow regulation in skeletal muscle. Capillaries are embedded within the endomysium, are oriented parallel to the muscle fiber and are dependent on the oxidative capacity of the muscle; each muscle fiber is surrounded by several capillaries. Within the rat
spinotrapezius muscle, arterioles decrease in diameter with branching and range from 45 - 65μm within the primary arterioles to 10 - 18μm in the tertiary arterioles (Lash & Bohlen, 1992).

During periods of muscle activity, cardiovascular changes occur in response to increased metabolic demands, and depending on muscle fiber type and motor unit recruitment patterns, functional hyperemia and oxygen consumption of active muscles can exceed resting values by as much as 10x to 50x (Ordway, Floyd, Longhurst, & Mitchell, 1984; Segal & Kurjiaka, 1995). To provide fuel for muscle contractions, ATP is hydrolyzed, which requires large amounts of oxygen. As such, intracellular O₂ pressure decreases, and terminal arterioles dilate as a negative feedback control to the decrease in oxygen concentration.

Dilatation is a complex process that occurs when there is a substantial increase in locally formed vasodilators, such that it is able to overcome vasoconstrictor signals. Vasoconstrictor signals are evoked primarily by increased sympathetic drive to increase total peripheral vascular resistance and increase blood pressure (Lash & Bohlen, 1992; Nyberg, Gliemann, & Hellsten, 2015). Various molecules and vasomotor responses are involved, including release of nitric oxide and prostacyclin by endothelial cells and cell-to-cell communication within and among branches of the resistance network (Bagher & Segal, 2011; Hudlicka, 2011; Korthuis, 2011; Lash & Bohlen, 1992). Dilation of terminal arterioles increases capillary perfusion, allowing more gas and nutrient exchange. The amount of blood flow to active muscle increases with exercise intensity and is enabled through a mechanism termed ascending vasodilatation. Ascending vasodilatation involves the progression of smooth muscle relaxation from the distal branches of the arteriolar network, though intermediate and proximal arteriolar segments, and into the arterial supply proximal to the muscle (Bagher & Segal, 2011). The vasodilatory response beginning at the microcirculatory level rapidly spreads to the feed arteries that control blood flow into the...
arteriolar network. This coordination of vasodilatation allows for substantive changes in blood flow during times of need (Bagher & Segal, 2011).

1.3.3 Adaptation Models

Blood vessels undergo three primary forms of remodeling - hypotrophic, eutrophic, and hypertrophic remodeling (Mulvany, 1999). Vascular remodeling refers to a variety of changes in dimensions and composition of the blood vessel that occur in response to both acute and chronic stimuli. Acute stimuli are caused primarily by sympathetic drive to smooth muscle cells within the medial layer, and chronic stimuli are often caused by changes in blood flow and pressure, which alter the hemodynamic environment (Silver & Vita, 2006). Hypotrophic, eutrophic, and hypertrophic remodeling refer to vascular changes that exhibit a decrease, no change, or an increase in the amount of wall material, respectively. These three forms of remodeling may be described as inward or outward. Inward remodeling causes a reduction in lumen diameter while outward remodeling causes an increase in lumen diameter. Studies on essential hypertension and renovascular hypertension have primarily shown inward eutrophic remodeling and inward hypertrophic remodeling (Humphrey, 2008a; Intengan & Schiffrin, 2001; Nyberg et al., 2015).

Inward eutrophic remodeling involves a reduced passive luminal diameter with no significant change in wall cross-sectional area and is associated with increased incidence of life-threatening cardiovascular events (Martinez-Lemus, Zhao, Galinanes, & Boone, 2011).

Wall shear stress and wall tension are the two main hemodynamic forces acting on vascular walls to cause structural adaptations. Wall shear stress and wall tension are caused by the flowing blood and intraluminal pressure, respectively. The Hagen – Poiseuille equation for blood flow within an inelastic, cylindrical, and straight vessel (Equation 1) is used to model wall shear stress. Wall shear stress ($\tau$) is directly related to blood viscosity ($\mu$) and mean volumetric
flow rate (Q) and inversely related to the third power of vessel diameter (d) (J. E. Moore Jr, Xu, Glagov, Zarins, & Ku, 1994). Wall tension (σ) is directly related to intraluminal pressure (P), vessel radius (r), and inversely related to wall thickness (t) (Glagov, Vito, Giddens, & Zarins, 1992) by Laplace’s Law (Equation 2).

**Equation 1:** *Hagen – Poiseuille*  \[ \tau = \frac{32\mu Q}{\pi d^4} \]

**Equation 2:** *Laplace’s Law*  \[ \sigma = \frac{P r}{t} \]

Blood vessels adapt structurally to changes in wall shear stress in order to maintain a preferred baseline range of physiological shear stresses. Wall shear stress is primarily applied to the intima as the intima is in direct contact with the flowing blood. In response to an acute increase in wall shear stress, endothelial cells upregulate endothelial nitric oxide synthase to increase production of nitric oxide (Humphrey, 2008a). Nitric oxide is a vasodilatory molecule that causes relaxation of smooth muscle cells, thereby leads to an increase in diameter. As shown in Equation 1, an increase in vessel diameter significantly reduces wall shear stress. Once wall shear stress returns to baseline levels, nitric oxide production decreases, and the blood vessel returns to baseline contractile state. If the increased flow rate is chronically sustained, prolonged increase in nitric oxide production induces cellular proliferation and matrix reorganization (Humphrey, 2008a). Abnormal levels of wall shear stress have been implicated in many clinical cardiovascular diseases, including atherosclerosis, stroke, thrombosis, and hypertension (Chiu & Chien, 2011; Jeong, Lee, & Rosenson, 2014; Q. Liu, Mirc, & Fu, 2008; Wootton & Ku, 1999; Xie, Wang, Zhu, & Zhou, 2014).

Similarly, changes in wall tension induce vascular remodeling in a manner that restores baseline levels. Wall tension is the normal stress in the wall circumferential direction due to
intraluminal pressure and is distributed to all three layers of the vessel wall (Papaioannou & Stefanadis, 2005). In response to an acute increase in wall tension due to increased pressure, the blood vessel constricts, which results in a decrease in vessel radius and, subsequently, lowers wall tension to baseline levels (Humphrey, 2008b; Mulvany, 1999). Chronic changes in wall tension induce remodeling of the vascular wall primarily within the tunica media. Wall stress is wall tension divided by thickness. Blood vessels have a preferred range of wall stress and will hypertrophy or atrophy to restore baseline levels. Tunica media is oriented in lamellar units, concentric layers of familiarly uniform composition, comprised of smooth muscle and fibrous proteins elastin and collagen. In cases of chronically elevated wall tension, there is proliferation of lamellar units, which increases the overall thickness of the vascular wall and, thereby, reduces wall stress (Wolinsky & Glagov, 1967). Conversely, when wall tension is chronically decreased, there is loss in lamellar units, which decreases wall thickness and restores wall stress. (Fedoroff, Burkholder, Gotlieb, & Langille, 1991).

### 1.4 RELEVANT FINDINGS FROM PREVIOUS STUDIES

#### 1.4.1 Vascular Changes Following Spinal Cord Injury

Although microcirculatory effects within in the spinal cord following SCI are well-studied, there is paucity of research on changes in peripheral microcirculation following SCI. Most studies on the cardiovascular system after SCI have focused on central cardiovascular control, and the studies investigating peripheral circulation have focused on conduit arteries.

Structural remodeling of the large conduit arteries below the level of injury following SCI may be primarily driven by reduced metabolic demands due to muscle inactivity. Inward remodeling of the femoral artery occurs with a decrease in arterial diameter of approximately 30 – 50% and a reduction in resting blood flow by approximately 30 – 40% (De Groot et al., 2003;
de Groot, Bleeker, van Kuppevelt, van der Woude, & Hopman, 2006; Schmidt-Trucksass et al., 2000). Large blood vessel remodeling appears to be complete within 6 weeks after injury (De Groot et al., 2003). In contrast to lower limb vasculature, upper limb vasculature appears to be unchanged. Common carotid artery and brachial artery diameters are similar between individuals with SCI and able-bodied individuals (De Groot et al., 2003; de Groot et al., 2006; Stoner et al., 2005). There are no differences in femoral artery diameter and maximal blood flow per unit muscle volume between individuals with SCI and able-bodied controls after accounting for the effect of reduced metabolic demand by normalizing femoral artery diameter to leg muscle volume (Olive, Dudley, & McCully, 2003).

Similarly, changes in sympathetic tone are likely to promote structural adaptations of the vascular wall. Decreased sympathetic innervation of blood vessels is likely to cause blood vessels to lose baseline contractility and induce vascular atrophy, while increased sympathetic tone may maintain or promote smooth muscle cell hypertrophy. Given the changes in sympathetic tone following SCI, blood vessels may exhibit histological changes.

To our knowledge, only two previous studies have examined effects of SCI on microvascular. Based on laser Doppler flowmetry after complete high and low thoracic spinal cord transection in rats (Guizar-Sahagun et al., 2004), there is no significant difference in microvascular blood flow of the hindlimb footpad and kidney between control and transected groups, which suggests the microvascular network in hindlimbs may be intact in SCI (Guizar-Sahagun et al., 2004). Further, histological analysis used to investigate structural remodeling of rat superior mesenteric arteries after T3 spinal cord transection and a survival period of 4 weeks (Moniri, 2012) indicated no significant differences in medial thickness and wall to lumen ratio between control and SCI mesenteric arteries. However, there is a significant increase in medial
thickness and wall to lumen ratio when repetitive colorectal distension is combined with SCI (Moniri, 2012). Note that repetitive colorectal distension is an experimental method used to induce autonomic dysreflexia. These findings suggest disruptions in sympathetic activity alone may not induce structural remodeling. Structural adaptations are likely due to secondary cardiovascular complications, such as autonomic dysreflexia and orthostatic hypotension.

After SCI, there are changes in the hemodynamic forces exerted on the vascular walls, which are likely to induce structural remodeling. Individuals with SCI exhibit increases in femoral artery wall shear stress of approximately 50 – 100% compared to able-bodied controls (C. R. L. Boot, van Langen, & Hopman, 2003; De Groot et al., 2003; Schmidt-Trucksass et al., 2000). Both tetraplegics and paraplegics show similar increased shear stress levels (C. R. L. Boot et al., 2003), which suggests the increase in shear stress is likely due to physical inactivity rather than changes of sympathetic innervation as injury level causes differential loss of sympathetic innervation (West et al., 2013). It is postulated that inactivity leads to a reduction in vessel diameter and an increase in blood velocity, which increases wall shear stress as flow is maintained to pre-injury levels, thereby inducing vascular remodeling (Thijssen et al., 2009).

Flow-mediated dilation measures the ability of blood vessels to accommodate increases in blood flow by altering vessel diameter and is commonly used to assess endothelial dysfunction (Raitakari & Celermajer, 2000). Ability to modulate vessel diameter in response to changes in flow is dependent upon proper nitric oxide release by endothelial cells to stimulate smooth muscle cell relaxation. Flow-mediated dilation is preserved below the injury and reduced above the injury in individuals with SCI compared to able-bodied controls (de Groot, Poelkens, Kooijman, & Hopman, 2004; Stoner et al., 2005). However, changes in arterial structure and wall shear stress complicate efforts to elucidate mechanisms responsible for flow-mediated
dilation in SCI and the use of flow-mediated dilation as an indicator of endothelial function within deconditioned limbs has yet to be validated, so the functional importance of the flow-mediated dilation findings is unclear (West et al., 2013).

Despite some inconsistencies, most studies show increased peripheral vascular resistance after SCI (Groothuis, Boot, Houtman, van Langen, & Hopman, 2005; Groothuis et al., 2010; Hopman, Groothuis, Flendrie, Gerrits, & Houtman, 2002; Kooijman, Rongen, Smits, & Hopman, 2003). Alpha-adrenergic tone is preserved in femoral arteries after SCI (Kooijman et al., 2003), indicating that the mechanism responsible for increased vascular resistance does not seem to be related to loss of centrally mediated sympathetic tonic control. Increased vascular resistance may be related to the spontaneous recovery of spinal reflexes and recovery of sympathetic tone with time after injury. In addition, hypersensitivity to vasoconstrictors from changes in the vasoconstrictor pathways post injury is another likely contributor of increased vascular resistance (West et al., 2013). Sympathetic hypoactivity after high-level SCI causes reduced circulatory levels of epinephrine and norepinephrine and to compensate, blood vessels may develop hypersensitivity to vasoconstrictor substances (Mathias, Frankel, Christensen, & Spalding, 1976; McLachlan, 2007b; Schmidt-Trucksass et al., 2000; Teasell et al., 2000).

Increased peripheral resistance following SCI is somewhat surprising as results from a study on the effect of acute denervation on rat skeletal muscle microcirculation suggest that loss of supraspinal sympathetic control would cause increased peripheral blood vessel diameter and, thereby, decrease peripheral vascular resistance (Chen, Seaber, Bossen, & Urbaniak, 1991). However, it is important to highlight the difference between denervation/transection studies and contusion studies, as there is complete loss of centrally-mediated innervation in denervation/transection studies. Some level of sympathetic innervation is preserved in contusion
injuries. Another important note is that these findings are all in large conduit arteries. Given the importance of microvessels to vascular resistance, additional microcirculation studies are likely to be impactful.

1.4.2 Vascular Effects of Muscle Disuse

Various inactivity models, including hindlimb unloading and bed rest, are used to explore the effect of muscle disuse. Physical inactivity affects vascular structure and is associated with reduced vessel diameter, lowered blood flow, impaired endothelial function, increased wall shear stress, increased mean red blood cell velocity, and higher vascular resistance (Bleeker et al., 2005; Nyberg et al., 2015; Thijssen et al., 2009; Trappe et al., 2009). Physical inactivity is also associated with absence of reactive hyperemia, which refers to a transient increase in organ blood flow that occurs following a brief period of ischemia and is indicative of autoregulation, the ability to maintain a constant blood flow despite changes in perfusion pressure (Higashi et al., 1999). Femoral artery diameter decreases by approximately 13% and 17% after 25 and 52 days of inactivity, respectively, in a human bed rest (Bleeker et al., 2005). However, there are some discrepancies. Severe disuse of rat extensor digitorum longus muscle induced by a two-week long application of tetrodotoxin, a potent neurotoxin that inhibits firing of action potentials in nerves, is not associated with capillary loss, despite a 51.6% decrease in fiber cross sectional area (Tyml, Mathieu-Costello, & Noble, 1995). When corrected for muscle atrophy, the unchanged absolute number of capillaries equated to an increase in capillary density of approximately 139%.

Similarly, exercise training induces increased vascularization. An increase in regional blood flow capacity among trained muscle fibers is linked in part to increased arteriolar density (Laughlin et al., 2006). Endurance exercise training induces an increase in arteriolar density in
both red and white portions of the gastrocnemius muscle in rats (Laughlin et al., 2006). Interestingly, interval sprint training does not alter arteriolar density in either the red or white portions of the gastrocnemius muscle. Aerobic exercise training increases oxidative capacity and capillary density in rat skeletal muscle (Lash & Bohlen, 1992).

Exercise training also improves vascular function in various pathologies (Nyberg et al., 2015). Hypertension and diabetes result in and are exacerbated by eutrophic remodeling of conduit and small resistance blood vessels characterized by increased medial thickness, reduced lumen, and increased media to lumen ratio (Intengan & Schiffrin, 2001; Mulvany, 1999). Exercise training attenuates arterial remodeling in spontaneously hypertensive rats (Gu et al., 2013), increases capillary density in humans (Hansen, Nielsen, Saltin, & Hellsten, 2010), and improves vascular function by enhancing vasodilator formation and reducing levels of vasoconstrictors and reactive oxygen species (Nyberg et al., 2015). Due to lower muscle activity levels, there are likely to be structural changes in the vasculature of skeletal muscle due to decreased muscle activity following SCI.

1.4.3 Unexplained Incidence of Cardiovascular Disease in Chronic SCI

Over the last decade, there have been major changes in morbidity and mortality in the SCI population. Historically, renal and pulmonary diseases have been leading causes of mortality among individuals with SCI (Devivo, 2012). Due to medical advancements in providing acute care and treating renal and pulmonary complications, cardiovascular disease has emerged to become the leading cause of mortality (Cragg et al., 2013; Cragg et al., 2013; Gondim et al., 2004).

There is a higher prevalence of cardiovascular disease among individuals with SCI compared to the able-bodied population (Krum et al., 1992; Myers et al., 2007; Phillips et al.,
1998; West et al., 2013) that is not fully explained by traditional risk factors, such as physical inactivity, cholesterol level, resting BP, glucose intolerance, and smoking status (Krum et al., 1992; Phillips et al., 1998; West et al., 2013). For instance, high thoracic or cervical SCI typically results in autonomic disturbances that cause low resting blood pressure, which is usually considered cardio-protective. However, individuals with high thoracic or cervical SCI exhibit a larger prevalence of cardiovascular disease compared to the able-bodied population (West et al., 2013). This suggests that there may be other effects of SCI not currently studied that contribute to the elevated risk of cardiovascular disease, irrespective of traditional risk factors.

1.5 SPECIFIC AIM: CHARACTERIZE HISTOLOGICAL CHANGES IN THE MICROVASCULATURE OF RAT SKELETAL MUSCLE FOLLOWING CHRONIC SPINAL CORD INJURY

We aim to determine whether there are histological changes in the microvasculature of rat skeletal muscle after SCI both above and below the level of injury. To our knowledge, microvascular changes after SCI have not been investigated. Findings will increase understanding of how SCI impacts peripheral vasculature, which may help elucidate why there is higher incidence of cardiovascular disease within the SCI population and identify new rehabilitation targets for functional recovery.

Chronic SCI will be induced in rats using a contusion injury, and two pairs of phasic and postural muscles, one set above and one set below injury, will be collected for histological analysis. Fibers and non-capillary microvessels in a mid-belly muscle cross section will be characterized in terms of myofiber cross sectional area, microvascular diameter, wall thickness, wall to lumen ratio, and wall cross sectional area. Statistical analyses will be conducted to compare the SCI and control groups. We expect to see a significant difference between the
control and SCI groups below the level of injury and no significant difference above the level of injury. Changes may be attributed to altered sympathetic tone and muscle disuse.

CHAPTER 2: HISTOLOGICAL CHANGES IN RAT SKELETAL MUSCLE MICROVASCULATURE AFTER CHRONIC SPINAL CORD INJURY

2.1 INTRODUCTION

The purpose of this study was to determine whether there are histological changes in the microvasculature of rat skeletal muscle following chronic spinal cord injury (SCI). Histological changes would indicate structural remodeling, which would likely impact muscle performance and cardiovascular health. Adequate blood circulation is critical to preventing muscle fatigue, and microvessels are the primary determinant of vascular resistance. Histological changes are likely due to changes in sympathetic tone and physical deconditioning following SCI. Improved understanding of how SCI impacts peripheral microvasculature may help explain why there is a higher incidence of cardiovascular disease in individuals with SCI compared to able-bodied individuals.

In order to identify morphological changes in the muscle vasculature after SCI, we quantified the vascular histology of forelimb and hindlimb muscles of rats following chronic contusion injury with a 90 day survival period. We hypothesized that histological changes occur within the microvasculature of rat skeletal muscle below the level of injury following SCI. Study results will improve our understanding of how SCI impacts peripheral vasculature.

2.2 METHODS

Ten female Wistar rats (275-300 grams) (Charles River Laboratories, Portage, MI) were used for this experiment. Rats were randomized into two experimental groups: control ($n=5$) or
SCI ($n=5$). The control group was naïve and not exposed to surgery or post-operative care. Euthanasia and tissue collection were identical for both groups. The SCI group was given a contusion injury to induce a severe spinal cord injury and then allowed to survive for 90 days. Histological analysis was performed on excised muscles. All procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, WI) and the Clement J. Zablocki Veterans Affairs Medical Center (Milwaukee, WI) under protocol number 5096-08.

**Experimental Procedures**

**Spinal Cord Injury Procedure**

All experimental procedures and animal housing took place in the Clement J. Zablocki VA Veterinary Medical Unit Building (Milwaukee, WI). Surgical procedures were conducted in a conventional laboratory setting using aseptic techniques that met local guidelines for rodent survival surgery.

SCI rats underwent a laminectomy followed by severe spinal cord injury. Rats were first anesthetized using inhalant isoflurane. Initial dose of isoflurane was allowed to be as high as 5%, with additional doses between 1% and 3% depending upon toe-pinch withdrawal and respiratory rate. Eyes of the rat were protected by administering ophthalmic ointment. Rats were then shaved, sterilized with a Betadine scrub, and secured to a surgical board. An incision was made over the dorsal mid-thoracic region, and the spinal cord and overlying dura were exposed by performing a laminectomy over T7-T9. The rats were then placed in an impactor (W.M. Keck Center for Collaborative Neuroscience, Rutgers, NJ), and a 10g rod was dropped from 50mm onto the dura at T8 with an impact velocity of 0.963 m/s. This impact causes severe SCI and produces paraplegia or high-grade paresis (Zhang, Fang, Chen, Gou, & Ding, 2014). Following
the injury, the muscle layer was closed with 3-0 Vicryl using a simple continuous stitch. Subsequently, the subcuticular layer was closed using the same suture and stitch pattern. Each rat was continuously monitored until full recovery from anesthesia.

Postoperative Care

The rats were placed on postoperative care for 48 hours immediately after injury. Postoperative care involved fluid therapy (lactated Ringer’s solution), bladder expression, a daily dose of enrofloxacin (10 mg/kg subcutaneous; Bayer Healthcare LLC; Shawnee Mission, KS), and bi-daily doses of carprofen (5mg/kg subcutaneous; Rimadyl; Zoetis, Inc.; Kalamazoo, MI). Enrofloxacin was used to prevent infection, and carprofen was used to alleviate pain. After the postoperative care period, fluid therapy was administered as needed, determined by skin turgor testing. Heating pads were provided for 6 hours post-operation. Veterinary staff were contacted and recommendations were followed when signs of pain and distress were evident beyond the three day treatment period. SCI rats were allowed to survive for 90 days after surgery followed by euthanasia. Rats were housed individually in standard rodent shoebox cages with no filter top during the survival period to assure optimal post-surgical monitoring and recovery.

Behavioral and Sensory Assessments

Open field walking was evaluated according to the Basso, Beattie, and Bresnahan (BBB) locomotion scale method one day after surgery and weekly afterwards during the 90-day survival period after injury (Basso, Beattie, & Bresnahan, 1995). Following standard BBB protocol, each rat was placed briefly on a flat, 1m by 0.5m surface and observed for 4 minutes. Special attention was paid to movement of the hindlimbs, along with tail position. Hindlimb function was scored on a scale of 0 to 21 with score of 21 equating normal gait and 0 equating flaccid paralysis.
Sensory function was evaluated weekly after the surgical procedure using the hotplate test. Each rat was placed on a warm surface heated to a maximum temperature of 50°C, and the time from placement to when the rat licked either hindlimb was recorded. To avoid injury to the rat, the rat was removed from the heated surface after 15 to 20 seconds if no response occurred. Better sensory function is characterized by shorter response time.

**Tissue Collection**

Both control and SCI groups underwent a non-survival, transcardial perfusion, conducted under a chemical hood. Beuthanasia (Merck Animal Health, Madison, NJ, at least 0.22 ml/kg and equivalent to 120 mg/kg or to effect) mixed with 1ml of heparin was used for euthanasia. When corneal and pain reflexes (e.g., toe-pincher reflex) were absent, the rat was restrained in a supine position with hindlimbs completely adducted and forelimbs partially abducted to 90°. A standard transcardial perfusion was conducted as previously described (Gage, Kipke, & Shain, 2012) with a modification of not making a cut in the left ventricle prior to insertion of the perfusion needle. The right atrium was cut to allow for fluids to flow out. The rat was flushed with phosphate-buffered saline (750ml) followed by a perfusion of 10% neutral buffered formalin (750ml). PBS flushes blood out of the body, and formalin performs a whole body fixation, which prevents tissue autolysis and decay by creating covalent bonds and chemical cross-links between proteins, maintaining cells in a biological state. Throughout the procedure, perfusion pressure was monitored using a handheld manometer (Pyle PDMM01, Pyle Audio Inc., Brooklyn, NY) connected to perfusion tubing, and pressure was maintained at a mean of 100mmHg throughout the procedure by adjusting the perfusion rate.

Once the perfusion was concluded, biceps brachii, triceps brachii, tibialis cranialis, and soleus were dissected from each limb. These muscles were selected to enable a comprehensive
analysis, as phasic and postural muscles might experience different changes, along with muscles above and below the level of injury. In the rat, triceps brachii and soleus are postural muscles that aid in maintenance of an upright posture by resisting gravity, while biceps brachii and tibialis cranialis are phasic muscles involved in locomotion. Biceps brachii and triceps brachii are located above the level of injury, while tibialis cranialis and soleus are located below the level of injury. Postural muscles below the level of injury are likely to be most impacted by SCI and experience the most structural remodeling. The limb was carefully skinned to expose the underlying musculature. Connective tissue was gently separated with forceps to isolate the target muscle. Prior to excision, both ends of the muscle were attached to a cotton swab using thread to ensure the muscle was fixed in a consistent physiologic length. Samples were immediately submersed in a formalin bath for a minimum of 48 hours to eliminate concerns regarding effectiveness of whole body fixation though a transcardial perfusion and to ensure complete fixation.

**Sample Preparation**

After adequate fixation as determined through visual assessment, the muscle was detached, cut at mid-belly orthogonal to the long axis, and processed to allow for paraffin infiltration and embedding. After dehydration through a series of graded ethanol reagents of progressively increasing concentrations ranging from 70% to 100%, the tissue was cleared with xylene and then embedded in paraffin in an orientation such that transverse sections of the muscle could be obtained. All processing steps were carried out at room temperature with exception of paraffin infiltration, which was conducted at 60°C. Four μm sections were cut with an automated microtome, floated on a warm water bath, and mounted on poly-1-lysine-coated glass slides. Slides were labeled and allowed to dry at 45°C overnight.
Verhoeff-van Gieson Elastin Staining

Slides were stained with the Verhoeff-van Gieson elastin stain according to previously published procedures (Luna, 1968) to allow visualization of the microvasculature. Verhoeff-van Gieson elastin stain is a histochemical stain commonly used to visualize elastin fibers, along with medial borders such as the internal and external elastic lamina, in larger arteries. This staining procedure is regressive in that it requires tissue sections to be first overstained and then differentiated. The tissue was stained with a hematoxylin stain of ferric chloride and iodine and then, differentiation was produced by using excess ferric chloride to disrupt the tissue-mordant dye complex. Because elastin has the strongest affinity of the iron-hematoxylin complex, elastic tissue retains dye longer than other tissue elements.

Data Collection

Digital images of the stained sections were obtained for qualitative and quantitative observations. The entire cross section of each muscle was examined and imaged using a Hamamatsu NanoZoomer (Hamamatsu; Shizuoka, Japan) set at 40X objective lens. No further adjustments or highlighting were performed. All microvessels present in the cross section were imaged using an NDP viewer (Hamamatsu; Shizuoka, Japan) at total magnification of 80X and spatial resolution of 0.113μm.

Myofiber Quantification

To assess muscle atrophy, the entire cross section was manually traced to measure muscle cross sectional area. Then, a 0.5 mm by 0.5 mm field from the center of the transverse muscle section was extracted, and all complete fibers present within the field were manually traced to measure myofiber cross sectional area in order to determine whether myofiber atrophy occurred.
Microvascular Quantification

**Assumptions**

The transverse cut was assumed to be perpendicular to the long axis of all microvessels, which may be invalid for some microvessels as the arterial tree within muscle consists of vessels oriented at various angles, ranging from 0 to 180 degrees, with respect to the muscle’s long axis.

**Algorithm Overview**

A custom-written MATLAB algorithm was created to perform segmentation and characterization of the microvessels. The algorithm was semi-automated and divided into three main parts - pre-processing, segmentation, and post-processing. Segmentation relied on a mean-intensity based region-growing segmentation algorithm written by Dirk-Jan Koon at the University of Twente in Enschede, Netherlands (© 2009), and post-processing used an edge-linking algorithm written by Peter Kovesi at the University of Western Australia in Perth, Australia (© 1996-2013).

Pre-processing included image cropping, background elimination, image inversion and normalization, and elimination of residual red blood cells. Each microvessel image was cropped to a region enclosing the microvessel in order to reduce memory requirements and increase algorithm efficiency. Red and blue components of seed pixels representative of non-vascular background tissue were used to calculate a range of intensity values to decrease background noise. Pixels with sums of red and blue components falling within this range were set to maximum intensity (white). The resulting image was inverted and normalized to adhere to the image processing convention of foreground appearing light and account for inter-sample variation of staining intensities. To eliminate residual red-blood cells, they were segmented using three seed points and Koon’s region-growing segmentation. Segmented red-blood cells were set to 0 (black).
Segmentation relied on Koon’s region-growing algorithm to compute two masks – an outer mask, which encompassed the entire blood vessel, and an inner mask, which encompassed just the lumen. Three seed points representative of the vascular wall were selected on each pre-processed image. For the region growing, the inclusion criterion for pixels representing the vascular wall was set at 0.20, which means that pixels in a 9x9 neighborhood around each seed point, within a 20% window of region mean, were included as part of the region for the next iteration. Pixels in the expanded region became seed points for subsequent iterations and were used to update region mean. Segmentation was complete when successive iterations did not differ. All pixels higher than the final region mean were included as foreground. These three segmentations (one segmentation for each seed) were added to compute a final mask encompassing the entire blood vessel. The final mask was made binary by setting values greater than 0 to 1. The inner mask was obtained similarly with three seed points. Pixels indicative of the lumen were used as seed points, inclusion criterion was set at 0.10, so neighboring pixels within a 10% window of region mean were included in the region, and all pixels lower than final mean intensity were included as foreground in the final mask.

A post-processing step used edge detection and morphological information to remove extraneous objects in the masks. Kovesi’s edge-linking algorithm, which uses canny edge detection with a hysteresis threshold of 0.1, was used to compute an edge-linked image (Kovesi, 2015). Edge pixels were set to 0 in the mask image, which disconnected extraneous components. Built-in MATLAB functions were used to morphologically open the outer mask to remove thin connections and close inner mask to fill in small holes. The outer and inner masks were then used to segment the vascular wall.
For each blood vessel, outer and inner circumferences were measured and used to calculate diameter, wall thickness, wall to lumen ratio, and wall cross sectional area. Outer and lumen circumferences were assumed to be path lengths along the outer and inner boundaries of the vascular wall, and outer and lumen diameters were calculated using the equation relating circumference and diameter of a circle. Wall thickness was calculated using outer and lumen diameters (Equation 3). Wall to lumen ratio was calculated using wall thickness and lumen diameter, and wall cross sectional area was calculated using outer diameter and lumen diameter (Equation 4). Vessels smaller than 9μm in diameter were assumed to be capillaries and excluded from analysis.

**Equation 3: Wall thickness**

\[ t = \frac{D_{\text{outer}}}{2} - \frac{D_{\text{lumen}}}{2} \]

**Equation 4: Wall cross sectional area**

\[ \text{Wall CSA} = \left[ \pi \times \left( \frac{D_{\text{lumen}}}{2} \right)^2 \right] - \left[ \pi \times \left( \frac{D_{\text{outer}}}{2} \right)^2 \right] \]

**Statistical Analysis**

Muscles contained varying number of fibers and non-capillary microvessels. Using the Shapiro-Wilk test for normality, the data was determined to have a non-normal distribution. As such, the median was calculated for each sample and used for subsequent statistical analysis. Data points were considered outliers when the value was larger than the 3rd quartile by at least 1.5 times the interquartile range or smaller than the first quartile by at least 1.5 times the interquartile range. Group data for control and SCI were compared using the Kruskal Wallis test. Differences were considered statistically significant when p-value was less than 0.05.

**2.3 RESULTS**

The behavioral and sensory assessments were consistent with a severe SCI in the test group. All SCI animals exhibited low BBB scores following the surgery as shown in Figure 1. In
addition, the time to respond to the hotplate test decreased, suggesting that sensory function was impaired (Table 1).

Figure 1: BBB score over the 90 day survival period

BBB score for each SCI rat over the 90 day survival period. All rats exhibited low BBB scores immediately after injury, which then increased with time to stabilize at approximately 13 by week 3.
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Table 1: Sensory function over 90 day survival period. Values represent seconds to when rat licked either hindlimb in response to hotplate test.
Myofiber Analysis

SCI did not result in a significant difference in muscle cross sectional area or myofiber cross sectional area. Histological staining was conducted successfully and enabled characterization of the entire muscle cross section. Figure 2 shows representative muscle cross sections in which elastin is stained dark blue – black and connective tissue is stained pink.

Muscle cross sectional area was not significantly different between control and SCI groups ($p = 0.6752$, $0.7540$, $0.0758$, and $0.1172$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively) as shown in Figure 3. Figure 4 displays a representative manual tracing of myofibers in a 0.5mm by 0.5mm window centered on the muscle cross section. This segmentation was used to calculate the cross sectional area of each myofiber. Figure 5 shows the cross sectional area of all myofibers included in each sample. Each sample contained varying number of complete fibers as shown by the numbers in parentheses. Since the data was determined to be nonparametric, median was used to represent each sample in subsequent statistical analysis. Figure 6 summarizes the median of each sample, along with the group mean. Medians were consistent within and similar between control and SCI groups. Control and SCI myofiber cross sectional areas were not significantly different ($p = 0.0758$, $0.9168$, $0.0758$, and $0.9168$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively).
Figure 2: Representative muscle cross sections

Representative muscle cross sections from the soleus muscle in the control (A) and spinal cord injury (B) groups. Elastin is stained dark blue – black. The pink periphery of muscle represents connective tissue.
Figure 3: Muscle cross sectional area

Muscle cross sectional area in control (CTL) and SCI groups. The bars represent the mean, while the error bars represent the standard deviation. Differences were not significant for any muscle with $p > 0.05$.

Figure 4: Representative myofiber tracing

Representative myofiber tracing from the soleus in the control group. Each myofiber was manually traced using MATLAB and cross sectional area calculated based on the number of pixels.
Figure 5: Myofiber cross sectional area

Box and whisker plots of all myofiber cross sectional areas in μm² organized by sample. Myofiber cross sectional area was consistent between samples in each group and between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. In each box, the line represents the median, top edge represents 3<sup>rd</sup> quartile, bottom edge represents 1<sup>st</sup> quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied and is shown in parentheses (n = 5 samples per muscle per group).
Figure 6: Group myofiber cross sectional area

Median myofiber cross sectional area of each group in μm² ($n = 5$ samples per muscle per group). Myofiber cross sectional area was similar between the control and SCI groups with no significant differences ($p > 0.05$ for all muscles). CTL represents control muscle, and SCI represents spinal cord injury muscle, while BB, TB, TC, and S represent the biceps brachii, triceps brachii, tibialis cranialis, and soleus muscles, respectively. Each dot represents a sample median, and each bar represents the group mean.

Microvascular Analysis

SCI microvessels were not significantly different from control microvessels. All microvessels within the entire muscle cross-section were imaged and characterized in terms of luminal diameter, wall thickness, wall to lumen ratio, and wall cross sectional area. Representative microvessels are shown in Figure 7. Microvessel structure was not visually different between control and SCI groups. After identification, each blood vessel was segmented and characterized using MATLAB. Figure 8 shows an example output of the segmentation algorithm. Each sample contained varying number of microvessels, and the data were first assessed by examining distribution of all measurements and using box and whisker plots. Figure
9 shows the histogram and box and whisker plot of all wall to lumen ratio measurements within each sample for the soleus. Data were determined to be nonparametric, so median was used to represent each sample for subsequent statistical analysis. Figure 10 summarizes sample medians across all four metrics.

There was no significant difference between control and SCI groups of the same muscle for any of the four metrics. Control and SCI microvascular diameters were not significantly different with $p = 0.9168, 0.7540, 0.2506,$ and $0.2506$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively. Differences between control and SCI microvascular wall thickness were not significant with $p = 0.7540, 0.6015, 0.6015,$ and $0.1172$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively. Control and SCI microvascular wall to lumen ratios were not significantly different with $p = 0.6015, 0.7540, 0.1745,$ and $0.9168$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively. Differences between control and SCI microvascular wall cross sectional area were not significant with $p = 0.7540, 0.7540, 0.4647,$ and $0.0758$ for triceps brachii, biceps brachii, soleus, and tibialis cranialis, respectively.

**Figure 7:** *Representative microvessels*

Representative microvessels from the biceps brachii muscle in the control (A) and SCI (B) groups. Blue arrows indicate arterioles, while red arrows indicate venules.
Figure 8: Control biceps brachii microvessel segmentation

Representative steps involved in the segmentation of a microvessel. Masks were used to extract boundaries, and edge linking was used as a technique to refine the boundaries.
Figure 9: Soleus wall to lumen ratio measurements for each sample

Wall to lumen ratio measurements for each sample in the soleus muscle (n = 5 samples per group) in both histogram (A) and bar and whisker plot (B). Distribution of wall to lumen ratio was similar between control and spinal cord injury groups. CTL represents control, and SCI represents spinal cord injury. Each dot represents a single measurement, and number of measurements in each sample is shown in parentheses. In each box, the line represents the median, top edge represents 3rd quartile, bottom edge represents 1st quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied and is shown in parentheses (n = 5 samples per muscle per group).
Median diameter (A), wall thickness (B), wall to lumen ratio (C), and wall cross sectional area (D) for control and SCI samples (n = 5 samples per muscle per group). Differences between control and SCI groups were not significant for any of the four metrics (p > 0.05). Each dot represents a sample median, and each bar represents the group mean. CTL represents control muscle, and SCI represents spinal cord injury muscle. BB, TB, TC, and S represent biceps brachii, triceps brachii, tibialis cranialis, and soleus, respectively.

2.4 DISCUSSION

Review of Specific Aim and Summary of Major Findings

We found no significant histological changes in skeletal muscle and microvasculature of skeletal muscle following contusion spinal cord injury. Specifically, the SCI group did not exhibit a decrease in myofiber cross sectional area, microvascular diameter, wall thickness, wall to lumen ratio, or wall cross sectional area compared to controls. The findings indicated maintenance of muscle and microvasculature following SCI in this experimental model.
Lack of Changes in Myofibers

After human SCI, there are significant changes in morphology and contractility of muscles below the level of injury. Beginning 4 to 7 months post injury, there is a shift from normal type I slow and type II fast fibers to predominantly type II fast glycolytic fibers (Biering-Sorensen et al., 2009; Burnham et al., 1997). The proportion of slow myosin heavy chain isomers progressively decreases while the proportion of hybrid fibers co-expressing both slow and fast myosin heavy chain isomers steadily increases (Shields, 2002). The increase in fast myosin isomers results in faster muscle contractile speed and physiological properties consistent with fast motor units, which leads to higher muscle fatigability (Shields, 2002). With reduced activity and unloading, muscular atrophy below the level of injury begins to occur within the first months as characterized by decreased fiber diameter, cross sectional area, and volume (Fong et al., 2009; Shields, 2002). Six weeks after injury, mean SCI muscle cross sectional areas are 18% to 46% lower than controls (Castro et al., 1999).

Similar muscular adaptations occur in rats following SCI with some key differences, dependent on the injury model used. Spinal transection injury models are widely used to study axonal regeneration following SCI as transaction allows for a reproducible, complete injury (Ye et al., 2013). Muscles in transection studies exhibit similar atrophy as seen in human SCI muscles; the soleus muscle is 45% smaller than controls three months post injury (Talmadge, Roy, Caiozzo, & Edgerton, 2002). However, most human SCIs do not involve cord transection and are caused by transient compression or contusion (Bunge, Puckett, Becerra, Marcillo, & Quencer, 1993). Contusion injury models more closely reproduce important histopathological features observed in clinical traumatic SCI cases and are widely used to characterize neuromuscular, pathophysiological, and functional changes after SCI (Gregory, Vandenborne,
In contusion studies, there is also significant atrophy in hindlimb skeletal muscles immediately following injury, similar to human SCI. However, atrophy peaks one week post SCI and is completely reversed four weeks post SCI through spontaneous recovery (Hutchinson, Linderman, & Basso, 2001; M. Liu et al., 2008). The discrepancy in muscle properties between human SCI and the rat contusion injury model might be attributed to differences in spinal circuits that generate and control locomotion in rats and humans (Y. P. Gerasimenko, Makarovskii, & Nikitin, 2002; Y. Gerasimenko, Roy, & Edgerton, 2007). There is a higher level of locomotor recovery and hindlimb loading in rats after SCI compared to corresponding injury in humans, and the skeletal musculature is highly sensitive to the level of neuromuscular activity (Fong et al., 2009). When cast immobilization is combined with contusion SCI in order to limit hindlimb loading, there is greater loss in muscle size, muscle mass, and force production and prevention of spontaneous recovery (Ye et al., 2013).

Although often described as a debilitating complication of SCI, spasticity has mixed effects and might help maintain muscle mass (Mclellan, 1981; Parziale, Akelman, & Herz, 1993). Spasticity is an involuntary muscle activity resulting from injury to upper motor neurons within the central nervous system. Symptoms include spasms, hyperreflexia, clonus, co-contraction, increased resistance to passive stretch, and development of joint contractures, which are expressed as reduced joint range of motion and deformity due to changes in muscle and joint tissues (Adams & Hicks, 2005; McDonald, Kevin Garrison, & Schmit, 2005; Mclellan, 1981). The presence and degree of spasticity depend on the location and extent of injury, with higher and more complete lesions having most risk of developing spasticity (Adams & Hicks, 2005). Spasticity negatively impacts individuals with chronic SCI by interfering with mobility, transfer,
self-care, and activities of daily living. However, involuntary activity present in spasticity might help maintain muscle following SCI (Dhindsa, Merring, Brandt, Tanaka, & Griffin, 2011; Roy et al., 1999). Spastic muscle activity preserves some normal muscle fiber type composition and contractile properties, despite affected muscles remaining more fatigable compared to non-affected muscles (Roy et al., 1999). Muscle activation in the form of involuntary spasticity is associated with maintained muscle size and quality, and higher spasticity scores are associated with larger upper and lower leg muscle area after chronic SCI (Eser, Frotzler, Zehnder, Schiessl, & Denoth, 2005; Gorgey & Dudley, 2008; C. D. Moore et al., 2015). In addition, light to moderate spasticity might aid in helping individuals with lower limb paresis to attain standing function (Adams & Hicks, 2005).

In this study, sufficient muscle activity may have been preserved after injury to maintain muscle integrity. There was a sharp decrease in BBB scores immediately following injury which then began to increase one week post injury to stabilize in range from 10 to 13 by week 3 for the remainder of the 90 day survival period. This indicated progressive recovery in locomotion, consistent with spontaneous recovery of locomotion following SCI. Via visual inspection, the rats were able to move around well in the cage a few weeks following injury. Skeletal musculature is highly sensitive to neuromuscular activity, the levels of activation and loading imposed on the muscles (Edgerton & Roy, 1994; Edgerton, Roy, Allen, & Monti, 2002). In a rat model of spinal cord isolation, as little as one minute of brief, high-load isometric contractions per day is sufficient to ameliorate inactivity-induced adaptations in the rat gastrocnemius muscle, including loss of mass and maximum tetanic tension and the shift from lower to faster myofiber types (Kim et al., 2007). These findings suggest the lack of muscle loss was due to recovery of sufficient locomotion over the survival period simulating chronic SCI.
**Lack of Changes in Microvasculature**

The maintenance of smooth muscle seen in this study might be related to intact peripheral sympathetic activity. Findings from previous studies, including one conducted by our research group, suggest normalization of peripheral sympathetic activity following SCI. Garrison *et al.* (2008) investigated the interaction of autonomic and motor reflexes in chronic human spinal cord injury. The group compared sympathetic reflex responses of individuals with high-level (T5 and above) and low-level (below T5) injuries to a noxious electrocutaneous stimulus applied to the skin at the medial arch of the right foot. Lower extremity vascular conductance changes are similar between high and low level SCI for sympathetic reflex responses obtained through controlled noxious electrocutaneous stimulation, which suggests the magnitude of local sympathetic reflexes are similar in SCI, regardless of the level of injury (Garrison, Ng, & Schmit, 2008). In chronic human SCI, peripheral afferent stimulation activates decentralized sympathetic nerves below the lesion and causes marked increase in noradrenaline spillover in the legs and pronounced end organ response, which indicates some preserved neuronal function (Karlsson, Friberg, Lonnroth, Sullivan, & Elam, 1998b). Microneurographic recordings of action potentials from individual muscle nerve sympathetic fibers show sparse ongoing spontaneous impulse discharges (Wallin et al., 2014). Given these findings, recovery of peripheral sympathetic tone is likely a part of the neuroplasticity and remodeling occurring over time following SCI.

The lack of muscle atrophy likely impacts the lack of vascular changes, because vascular growth and metabolic activity are related. In tissues where the vasculature primarily serves nutritive function, which includes skeletal muscles investigated in this study, changes in metabolic activity cause secondary proportional changes in blood vessel growth or regression.
In a bed rest inactivity model, diameter of the common femoral artery decreases 13% and 17% after 25 and 52 days of bed rest, respectively (Bleeker et al., 2005). Conversely, treadmill training results in an approximately 20% increase in the passive diameter of primary and secondary arterioles in the rat spinotrapezius muscle (Lash & Bohlen, 1992). Absolute values of femoral artery diameter and blood flow are lower in individuals with chronic SCI compared to able-bodied controls (C. R. Boot, Groothuis, Van Langen, & Hopman, 2002; Hopman, van Asten, & Oeseburg, 1996; Stoner et al., 2005). However, the decreases are negated when diameter and blood flow values are normalized by muscle volume (Olive et al., 2003). These findings suggest the lack of vascular adaptations present in this study is likely linked to lack of muscular changes.

The lack of vascular adaptations in this study is consistent with findings from a previous histological study investigating superior mesenteric arteries after T3 spinal cord transaction (Moniri, 2012). Medial thickness and wall to lumen ratio of rat superior mesenteric arteries are not significantly different between SCI and control. However, when repetitive colorectal distension is added in addition to SCI, there are significant increases in both medial thickness and wall to lumen ratio compared to SCI alone and control groups (Moniri, 2012). Repetitive colorectal distension is an experimental technique used to induce autonomic dysreflexia, in which sympathetic drive to the mesenteric arterioles is increased and vasoconstriction occurs. This suggests structural remodeling occurs as a result of change in sympathetic tone and sympathetic changes following acute SCI may normalize over time in chronic SCI.

In summary, the lack of muscular and lack of vascular adaptations in this study were not as we initially hypothesized. We postulate that the lack of both muscular and vascular changes
are related. The lack of vascular adaptations was likely due to both maintenance of the muscle and normalization of sympathetic drive below the level of injury in chronic SCI.

**Relationship to Previous Work & Unique Contribution**

To the best of our knowledge, this study is the first to investigate histological changes in the microvasculature of rat skeletal muscles following SCI. The findings suggest muscle and microvasculature are maintained after contusion SCI in the rat. Spontaneous recovery of muscle activity associated with locomotion may be sufficient in maintaining muscle. The level of sympathetic tone following SCI may be enough to maintain microvascular integrity, which supports previous studies showing preserved sympathetic tone below the level of injury (Garrison et al., 2008). This suggests disruptions in sympathetic tone due to SCI may normalize over time.

**Study Limitations**

Results from this study should be interpreted within the constraints of potential limitations, including sampling error characteristics of histological studies, lack of specific blood pressure measurements, and relatively low sample size.

The microvascular network within a muscle is diverse and consists of many branch points (Engelson, Skalak, & Schmid-Schonbein, 1985; Hudlicka, 2011). To be consistent, all samples were taken from muscle mid-belly orthogonal to the muscle’s long axis. However, branch points might not occur at the same location in every muscle, and one cross sectional section might not be indicative of what occurs throughout the muscle. Future studies should consider taking samples from multiple locations within the muscle to ensure comprehensive assessment of muscle vasculature.
The vascular remodeling might have been impacted by systemic blood pressure changes. Resting blood pressure measurements would be useful in providing a detailed assessment of each rat’s cardiovascular state and identifying whether there was low resting blood pressure, characteristic of individuals with SCI. This would allow better evaluation of the appropriateness of using a rat model to study cardiovascular changes following SCI.

The sample size of the current study was limited and less than typical of similar rat studies (Gu et al., 2013; Lash & Bohlen, 1992; Schmidt-Trucksass et al., 2000; Tyml et al., 1999). This study was meant to be exploratory and if there were differences, they would have been confirmed with a larger study.

2.5 CONCLUSION & FUTURE DIRECTIONS

Although this study suggested an absence of structural remodeling in microvasculature of rat skeletal muscle following SCI, this does not signify absence of functional changes in the peripheral vasculature. While structural changes are important, with many pathologies being associated with changes in microvascular structure (Chesnutt & Han, 2011; Del Corso et al., 1998; Dobrin, Schwarcz, & Baker, 1988; Hiroki, Miyashita, & Oda, 2002; Humphrey, 2008a; Mulvany, 1999; Nyberg et al., 2015), functional changes are of equal if not greater clinical importance. Altered sympathetic patterns and muscle activity following SCI might induce changes in molecular mechanisms regulating blood vessel reactivity. For instance, endothelial function might be impacted such that the vasculature does not respond properly to shear stress changes.

Future research includes assessing microvascular function following SCI and investigating histological changes in large conduit blood vessels. An example study involves extracting specific arteriolar segments from rat forelimbs and hindlimbs after SCI, conducting
functional tests at specified pressures to assess endothelial function, and conducting immunohistochemical staining to determine protein expression levels and smooth muscle cell phenotype within the tunica media. Functional testing on extracted arterioles may provide a more accurate assessment of arteriolar function and potentially illuminate how SCI impacts function of peripheral vasculature.
REFERENCES


APPENDIX

ADDITIONAL RESULTS

Number of total measurements in each sample

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<tr>
<th></th>
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<th>Triceps Brachii</th>
<th>Tibialis Cranialis</th>
<th>Soleus</th>
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The number of fibers present in each muscle section that were used for analysis. CTL represents control animal, and SCI represents spinal cord injury animal. There were 5 samples of each muscle in each group, but the number of fibers varied dependent on the sample as shown in the table.

Table 3. Number of microvessels in each muscle cross section

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<tr>
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The number of non-capillary microvessels present in each muscle section that were used for analysis. CTL represents control animal, and SCI represents spinal cord injury animal. There were 5 samples of each muscle in each group, but the number of microvessels varied dependent on the sample as shown in the table.

Histograms

Microvascular Diameter
Figure 11. Histograms: all diameter measurements

Histograms of diameter, in microns, organized by sample. Diameter was similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. Each dot represents a single measurement, and the number of measurements in each sample varied ($n = 5$ samples per muscle per group).
Microvascular Wall Thickness

Figure 12. Histograms: all wall thickness measurements

Histograms of wall thickness, in microns, organized by sample. Wall thickness was similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. Each dot represents a single measurement, and the number of measurements in each sample varied ($n = 5$ samples per muscle per group).
**Figure 13.** Histograms: all wall to lumen ratio measurements

Histograms of wall to lumen ratio, in percent, organized by sample. Wall-to-lumen ratio was similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. Each dot represents a single measurement, and the number of measurements in each sample varied ($n = 5$ samples per muscle per group).
**Microvascular Wall Cross Sectional Area**

**Figure 14. Histograms: all wall cross sectional area measurements**

Histograms of wall cross sectional area, in μm², organized by sample. Wall cross sectional area was similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. Each dot represents a single measurement, and the number of measurements in each sample varied (n = 5 samples per muscle per group).
Box and whisker plots of all measurements

Microvascular Diameter

Box and whisker plots of all diameter measurements, in microns, organized by sample. Diameter was consistent between samples in each group and similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. In each box, the line represents the median, top edge represents 3rd quartile, bottom edge represents 1st quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied (n = 5 samples per muscle per group).
Figure 16. Box and Whisker Plots: all wall thickness measurements

Box and whisker plots of all wall thickness measurements, in microns, organized by sample. Wall thickness was consistent between samples in each group and similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. In each box, the line represents the median, top edge represents 3rd quartile, bottom edge represents 1st quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied (n = 5 samples per muscle per group).
**Microvascular Wall to Lumen Ratio**

*Figure 17. Box and Whisker Plots: all wall to lumen ratio measurements*

Box and whisker plots of all wall to lumen ratio measurements, in percent, organized by sample. Wall-to-lumen ratio was consistent between samples in each group and similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. In each box, the line represents the median, top edge represents 3rd quartile, bottom edge represents 1st quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied (n = 5 samples per muscle per group).
**Microvascular Wall Cross Sectional Area**

Figure 18. *Box and Whisker Plots: all wall cross sectional area measurements*

Box and whisker plots of all wall cross sectional area measurements, in μm², organized by sample. Wall cross sectional area was consistent between samples in each group and similar between control and spinal cord injury muscles. CTL represents control muscle, and SCI represents spinal cord injury muscle. In each box, the line represents the median, top edge represents 3rd quartile, bottom edge represents 1st quartile, and whiskers represent the farthest points that are not considered outliers. Outliers are shown as crosses. The number of measurements in each sample varied (n = 5 samples per muscle per group).

**Kruskal Wallis Statistical Outputs**

*Muscle cross sectional area*

**Triceps brachii**

Table 4. Kruskal-Wallis test for triceps brachii muscle CSA

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Kruskal-Wallis Test results comparing triceps brachii muscle CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.6752, which means that there was no significant difference between control and spinal cord injury triceps brachii muscle CSA.

**Biceps brachii**

*Table 5. Kruskal-Wallis test for biceps brachii muscle CSA*

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Kruskal-Wallis Test results comparing biceps brachii muscle CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury biceps brachii muscle CSA.

**Soleus**

*Table 6. Kruskal-Wallis test for soleus muscle CSA*

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Kruskal-Wallis Test results comparing soleus muscle CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.0758, which means that there was no significant difference between control and spinal cord injury soleus muscle CSA.

**Tibialis cranialis**

*Table 7. Kruskal-Wallis test for tibialis cranialis muscle CSA*

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Kruskal-Wallis Test results comparing tibialis cranialis muscle CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.1172, which means that there was no significant difference between control and spinal cord injury tibialis cranialis muscle CSA.

Myofiber cross sectional area

**Triceps brachii**

Table 8. *Kruskal-Wallis test for triceps brachii myofiber CSA*

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Kruskal-Wallis Test results comparing triceps brachii myofiber CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.0758, which means that there was no significant difference between control and spinal cord injury triceps brachii myofiber CSA.

**Biceps brachii**

Table 9. *Kruskal-Wallis test for biceps brachii myofiber CSA*

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</table>

Kruskal-Wallis Test results comparing biceps brachii myofiber CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.9168, which means that there was no significant difference between control and spinal cord injury biceps brachii myofiber CSA.

**Soleus**

Table 10. *Kruskal-Wallis test for soleus myofiber CSA*

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>19.000</td>
<td>27.500</td>
<td>3.80000</td>
<td>-1.671</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>36.000</td>
<td>27.500</td>
<td>7.20000</td>
<td>1.671</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1527</td>
<td>1</td>
<td>0.0758</td>
</tr>
</tbody>
</table>
Kruskal-Wallis Test results comparing soleus myofiber CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.0758, which means that there was no significant difference between control and spinal cord injury soleus myofiber CSA.

**Table 11. Kruskal-Wallis test for tibialis cranialis myofiber CSA**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>28.000</td>
<td>27.500</td>
<td>5.60000</td>
<td>-0.000</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>27.000</td>
<td>27.500</td>
<td>5.40000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0109</td>
<td>1</td>
<td>0.9168</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing tibialis cranialis myofiber CSAs. The p (alpha level of 0.05) highlighted in yellow is 0.0758, which means that there was no significant difference between control and spinal cord injury tibialis cranialis myofiber CSA.

**Microvascular Diameter**

**Table 12. Kruskal-Wallis test for triceps brachii microvascular diameter**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>-0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0109</td>
<td>1</td>
<td>0.9168</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing triceps brachii microvascular diameter. The p (alpha level of 0.05) highlighted in yellow is 0.9168, which means that there was no significant difference between control and spinal cord injury triceps brachii microvascular diameter.

**Biceps brachii**

**Table 13. Kruskal-Wallis test for biceps brachii microvascular diameter**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>-0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0982</td>
<td>1</td>
<td>0.7540</td>
</tr>
</tbody>
</table>
Kruskal-Wallis Test results comparing biceps brachii microvascular diameter. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury biceps brachii microvascular diameter.

Soleus

Table 14. Kruskal-Wallis test for soleus microvascular diameter

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>33.000</td>
<td>27.500</td>
<td>6.60000</td>
<td>1.044</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>22.000</td>
<td>27.500</td>
<td>4.40000</td>
<td>-1.044</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3200</td>
<td>1</td>
<td><strong>0.2506</strong></td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing soleus microvascular diameter. The p (alpha level of 0.05) highlighted in yellow is 0.2506, which means that there was no significant difference between control and spinal cord injury soleus microvascular diameter.

Tibialis cranialis

Table 15. Kruskal-Wallis test for tibialis cranialis microvascular diameter

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>22.000</td>
<td>27.500</td>
<td>4.40000</td>
<td>-1.044</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>33.000</td>
<td>27.500</td>
<td>6.60000</td>
<td>1.044</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3200</td>
<td>1</td>
<td><strong>0.2506</strong></td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing tibialis cranialis microvascular diameter. The p (alpha level of 0.05) highlighted in yellow is 0.2506, which means that there was no significant difference between control and spinal cord injury tibialis cranialis microvascular diameter.

Microvascular Wall thickness

Triceps brachii

Table 16. Kruskal-Wallis test for triceps brachii microvascular wall thickness

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>-0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0982</td>
<td>1</td>
<td><strong>0.7540</strong></td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing triceps brachii microvascular wall thickness. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury triceps brachii microvascular wall thickness.
Kruskal-Wallis Test results comparing triceps brachii microvascular wall thickness. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury triceps brachii microvascular wall thickness.

Biceps brachii

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
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<td>25.000</td>
<td>27.500</td>
<td>5.00000</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>30.000</td>
<td>27.500</td>
<td>6.00000</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2727</td>
<td>1</td>
<td>0.6015</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing biceps brachii microvascular wall thickness. The p (alpha level of 0.05) highlighted in yellow is 0.6015, which means that there was no significant difference between control and spinal cord injury biceps brachii microvascular wall thickness.

Soleus

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>25.000</td>
<td>27.500</td>
<td>5.00000</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>30.000</td>
<td>27.500</td>
<td>6.00000</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2727</td>
<td>1</td>
<td>0.6015</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing soleus microvascular wall thickness. The p (alpha level of 0.05) highlighted in yellow is 0.6015, which means that there was no significant difference between control and spinal cord injury soleus microvascular wall thickness.

Tibialis cranialis

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>20.000</td>
<td>27.500</td>
<td>4.00000</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>35.000</td>
<td>27.500</td>
<td>7.00000</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4545</td>
<td>1</td>
<td>0.1172</td>
</tr>
</tbody>
</table>
Kruskal-Wallis Test results comparing tibialis cranialis microvascular wall thickness. The p (alpha level of 0.05) highlighted in yellow is 0.1172, which means that there was no significant difference between control and spinal cord injury tibialis cranialis microvascular wall thickness.

Microvascular Wall to Lumen ratio

**Triceps brachii**

**Table 20. Kruskal-Wallis test for triceps brachii microvascular wall to lumen ratio**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>30.000</td>
<td>27.500</td>
<td>6.00000</td>
<td>0.418</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>25.000</td>
<td>27.500</td>
<td>5.00000</td>
<td>-0.418</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2727</td>
<td>1</td>
<td>0.6015</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing triceps brachii microvascular wall to lumen ratio. The p (alpha level of 0.05) highlighted in yellow is 0.6015, which means that there was no significant difference between control and spinal cord injury triceps brachii microvascular wall to lumen ratio.

**Biceps brachii**

**Table 21. Kruskal-Wallis test for biceps brachii microvascular wall to lumen ratio**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>-0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0982</td>
<td>1</td>
<td>0.7540</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing biceps brachii microvascular wall to lumen ratio. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury biceps brachii microvascular wall to lumen ratio.

**Soleus**

**Table 22. Kruskal-Wallis test for soleus microvascular wall to lumen ratio**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>21.000</td>
<td>27.500</td>
<td>4.20000</td>
<td>-1.253</td>
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<tr>
<td>SCI</td>
<td>5</td>
<td>34.000</td>
<td>27.500</td>
<td>6.80000</td>
<td>1.253</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8436</td>
<td>1</td>
<td>0.1745</td>
</tr>
</tbody>
</table>
Kruskal-Wallis Test results comparing soleus microvascular wall to lumen ratio. The p (alpha level of 0.05) highlighted in yellow is 0.1745, which means that there was no significant difference between control and spinal cord injury soleus microvascular wall to lumen ratio.

*Tibialis cranialis*

**Table 23. Kruskal-Wallis test for tibialis cranialis microvascular wall to lumen ratio**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>27.000</td>
<td>27.500</td>
<td>5.40000</td>
<td>0.000</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>28.000</td>
<td>27.500</td>
<td>5.60000</td>
<td>-0.000</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0109</td>
<td>1</td>
<td>0.9168</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing tibialis cranialis microvascular wall to lumen ratio. The p (alpha level of 0.05) highlighted in yellow is 0.9168, which means that there was no significant difference between control and spinal cord injury tibialis cranialis microvascular wall to lumen ratio.

**Microvascular Wall cross sectional area**

*Triceps brachii*

**Table 24. Kruskal-Wallis test for triceps brachii microvascular wall CSA**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0982</td>
<td>1</td>
<td>0.7540</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test results comparing triceps brachii microvascular wall CSA. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury triceps brachii microvascular wall CSA.

*Biceps brachii*

**Table 25. Kruskal-Wallis test for biceps brachii microvascular wall CSA**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>26.000</td>
<td>27.500</td>
<td>5.20000</td>
<td>-0.209</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>29.000</td>
<td>27.500</td>
<td>5.80000</td>
<td>0.209</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0982</td>
<td>1</td>
<td>0.7540</td>
</tr>
</tbody>
</table>
Kruskal-Wallis Test results comparing biceps brachii microvascular wall CSA. The p (alpha level of 0.05) highlighted in yellow is 0.7540, which means that there was no significant difference between control and spinal cord injury biceps brachii microvascular wall CSA.

**Soleus**  
**Table 26. Kruskal-Wallis test for soleus microvascular wall CSA**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>31.000</td>
<td>27.500</td>
<td>6.20000</td>
<td>0.627</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>24.000</td>
<td>27.500</td>
<td>4.80000</td>
<td>-0.627</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation  
ChiSquare    DF    Prob>ChiSq  
0.5345       1    0.4647

Kruskal-Wallis Test results comparing soleus microvascular wall CSA. The p (alpha level of 0.05) highlighted in yellow is 0.4647, which means that there was no significant difference between control and spinal cord injury soleus microvascular wall CSA.

**Tibialis cranialis**  
**Table 27. Kruskal-Wallis test for tibialis cranialis microvascular wall CSA**

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Expected Score</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>5</td>
<td>19.000</td>
<td>27.500</td>
<td>3.80000</td>
<td>-1.671</td>
</tr>
<tr>
<td>SCI</td>
<td>5</td>
<td>36.000</td>
<td>27.500</td>
<td>7.20000</td>
<td>1.671</td>
</tr>
</tbody>
</table>

1-Way Test, ChiSquare Approximation  
ChiSquare    DF    Prob>ChiSq  
3.1527       1    0.0758

Kruskal-Wallis Test results comparing tibialis cranialis microvascular wall CSA. The p (alpha level of 0.05) highlighted in yellow is 0.0758, which means that there was no significant difference between control and spinal cord injury tibialis cranialis microvascular wall CSA.
function [ stats ] = AnalyzeVessel( image_in, outCrit, inCrit )

This function conducts quantification of blood vessels.

Input is an image containing a cross sectional view of a blood vessel.
Function first displays the image. User is be prompted to draw a small
box within the blood vessel wall to generate a seed image. Region
growing based on region mean is used to extract outer and inner masks.
Opening and closing techniques clean up the image. Connected component
analysis is used to select masks. Inner and outer perimeters and

cross- sectional area are computed.

Part 1. Pre-processing image cropping and background elimination -

% Image loading
figure, subplot(3,4,1), imagesc(image_in), axis image, colormap(gray),
title('Original'); % displays input image in gray scale
fprintf('
Please select a region encompassing the blood vessel. Left click on mouse and drag. Release when finished.\n'); % displays instructions

croppedIm(:,:,1) = imcrop((image_in(:,:,1)), position); % crops image
croppedIm(:,:,2) = imcrop((image_in(:,:,2)), position); % crops image
croppedIm(:,:,3) = imcrop((image_in(:,:,3)), position); % crops image

% Background elimination
fprintf('Starting background elimination'); % displays progress
fprintf('Select any number of points representative of background to be eliminated.\n'); % displays instructions
bkgd = impixel(croppedIm); % extracts RGB components of selected pixels
RplusBbkgd = (bkgd(:,1)+ bkgd(:,3)); % adds red and blue components
sm = min(RplusBbkgd); % computes min and max red and blue components to get range for background elimination
lg = max(RplusBbkgd);
RplusB = double(croppedIm(:,:,1)) + double(croppedIm(:,:,3)); % computes R+B for entire image
croppedR = croppedIm(:,:,1);
maxR = max(croppedR(:)); % determines max red intensity
croppedR((RplusB>(sm)) & (RplusB<(lg))) = maxR; % sets background pixels to lightest color
% Image inversion and normalization
croppedR = imcomplement(croppedR); % performs image inversion so blood vessel appears light to conform to convention of foreground appearing light
croppedRnorm = mat2gray(croppedR); % normalizes image to account for variation in staining
subplot(3,4,2), imagesc(croppedRnorm), axis image, colormap(gray),
title('Cropped & Normalized'); % displays cropped image

% Residual red-blood cell elimination
numRBC = input('
Starting residual RBC elimination... Enter number to be eliminated\n'); % reads in the user input
if numRBC ~= 0 % enters loop for red-blood cell elimination
    [n,m] = size(croppedRnorm); % calculates size
    RBCMask = zeros(n,m);
    answer = input('Do you want to manually eliminate red-blood cells? Reply "Y" or "N" ', 's'); % prompts for and reads in user input
    if answer == 'Y'
        for j=1:numRBC
            disp('Please trace the area to be removed.'); % displays instructions
            trace = imfreehand(); % enables ROI drawing tool
            jRBCMask = trace.createMask(); % creates outer mask from ROI drawn
            RBCMask = RBCMask + jRBCMask; % adds red-blood cell segmentations incrementally
        end
    else
        for j=1:numRBC
            fprintf('
Select 3 seed points on the residual RBC(s) to be eliminated.\n'); % displays instructions
            [RBCseedY, RBCseedX] = getpts(get(imshow(croppedRnorm), 'Parent')); % calls cursor to choose set of points as seed
            RBCseedX = round(RBCseedX); RBCseedY = round(RBCseedY); % rounds coordinates to avoid non-integer values
            [RBCMask1, ~] = regiongrowing(croppedRnorm, RBCseedX(1), RBCseedY(1), inCrit);
            [RBCMask2, ~] = regiongrowing(croppedRnorm, RBCseedX(2), RBCseedY(2), inCrit);
            [RBCMask3, ~] = regiongrowing(croppedRnorm, RBCseedX(3), RBCseedY(3), inCrit);
            jRBCMask = RBCMask1 + RBCMask2 + RBCMask3; % adds results of all 3 segmentations together
            RBCMask = RBCMask + jRBCMask;
        end
    end
    croppedRnorm(RBCMask~=0) = 0;
end

% Part 2. Mask Segmentation -
subplot(3,4,2), imagesc(croppedRnorm), axis image, colormap(gray),
title('Cropped & Normalized'); % displays image
fprintf('
Starting mask segmentation\n'); % displays progress
% obtains seed points for mask segmentations
fprintf('
Select 3 seed points to begin outer mask segmentation. \nLeft click on mouse to select, and press enter when finished.\n'); % displays instructions

%Note: row and col are switched in getpts for some odd reason
[outseedY, outseedX] = getpts(get(ismshow(croppedRnorm), 'Parent')); %calls
cursor to choose set of points as seed
outseedX = round(outseedX); outseedY = round(outseedY); %rounds coordinates
to avoid non-integer values
fprintf('
Select 3 seed points to begin inner mask segmentation.\nLeft click on mouse to select, and press enter when finished\n'); %displays instructions
%Note: row and col are switched in getpts for some odd reason
[inseedY, inseedX] = getpts(get(ismshow(croppedRnorm), 'Parent')); %calls
cursor to choose set of points as seed
inseedX = round(inseedX); inseedY = round(inseedY); %rounds coordinates to
avoid non-integer values

%Calls region growing function for 3 vascular wall mask segmentations
[outMask1, endRegMean] = regiongrowing(croppedRnorm, outseedX(1),
outseedY(1), outCrit);
outMask1 = or(outMask1, uint8(croppedRnorm >= endRegMean)); %includes pixels
intensity higher than mean intensity of segmented region
[outMask2, endRegMean] = regiongrowing(croppedRnorm, outseedX(2),
outseedY(2), outCrit);
outMask2 = or(outMask2, uint8(croppedRnorm >= endRegMean)); %includes pixels
intensity higher than mean intensity of segmented region
[outMask3, endRegMean] = regiongrowing(croppedRnorm, outseedX(3),
outseedY(3), outCrit);
outMask3 = or(outMask3, uint8(croppedRnorm >= endRegMean)); %includes pixels
intensity higher than mean intensity of segmented region
outMask = outMask1 + outMask2 + outMask3; %adds results of all 3
segmentations together
outMask = outMask > 0; %creates binary image

%Calls region growing function to perform 3 segmentations of lumen mask
[inMask1, endRegMean] = regiongrowing(croppedRnorm, inseedX(1), inseedY(1),
inCrit);
inMask1 = or(inMask1, uint8(croppedRnorm <= endRegMean)); %includes pixels
with intensity lower than mean intensity of segmented region
[inMask2, endRegMean] = regiongrowing(croppedRnorm, inseedX(2), inseedY(2),
inCrit);
inMask2 = or(inMask2, uint8(croppedRnorm <= endRegMean)); %includes pixels
with intensity lower than mean intensity of segmented region
[inMask3, endRegMean] = regiongrowing(croppedRnorm, inseedX(3), inseedY(3),
inCrit);
inMask3 = or(inMask3, uint8(croppedRnorm <= endRegMean)); %includes pixels
with intensity lower than mean intensity of segmented region
inMask = inMask1 + inMask2 + inMask3; %adds results of all 3 segmentations
together
inMask = inMask > 0; %creates binary image

%Displays results
subplot(3,4,3), imagesc(outMask), axis image, colormap(gray), title('Raw
Outer Mask'); %displays segmented image
subplot(3,4,4), imagesc(inMask), axis image, colormap(gray), title('Raw Inner
Mask'); %displays segmented image

%Part 3. Post-processing -
%Computes edge-linked image
% Beginning of code by Peter Kovesi
% Find edges using the Canny operator with hysteresis thresholds of 0.1
% and 0.2 with smoothing parameter sigma set to 1.
edgeim = edge(croppedRnorm, 'canny', [0.1 0.2], 1);

% Link edge pixels together into lists of sequential edge points, one
% list for each edge contour. Discard contours less than 10 pixels long.
[~, labelededgeim] = edgelink(edgeim, 10);

% End of code by Peter Kovesi

% Uses edge-linked image to clean up borders of masks
edgeIm = labelededgeim > 0; % determines edge image
outMask(edgeIm==1) = 0; % sets canny edge pixels to 0 in outer and inner mask
inMask(edgeIm==1) = 0;

% Opening to remove thin connections from outer mask and close thin gaps in
inner mask-
structEl = ones(3,3); % creates structuring element
openedOut = imopen(outMask, structEl); % performs image opening
closedIn = imclose(inMask, structEl); % performs image closing

% Connected component analysis to obtain outer mask
ccOut = bwlabel(openedOut, 4); % performs connect component analysis and yields labeled image
subplot(3,4,5), imagesc(ccOut), axis image, colormap(gray), title('Labeled + Opened Outer Mask'); % displays closed image
fprintf('
Please click anywhere on the connected component representing the outer mask. Press enter when you are done.
'); % displays instructions
[y, x] = getpts(get(imshow(ccOut), 'Parent')); % calls cursor to choose set of points as seed
y = round(y); x = round(x);
openedOut = ccOut == ccOut(x,y); % selects only that connected component

% Fill holes in segmented image for outer perimeter calculation
filledOut = imfill((imclose(openedOut, structEl)), 'holes'); % closing to get rid of added edges and filling closed to avoid unexpected errors in using regionprops
ccOut = bwlabel(filledOut, 4); % performs connect component analysis and yields labeled image
subplot(3,4,6), imagesc(ccOut), axis image, colormap(gray), title('Closed + Filled Outer Mask'); % displays closed image
fprintf('
Please click anywhere on the connected component representing the outer mask. Press enter when you are done.
'); % displays instructions
[y, x] = getpts(get(imshow(ccOut), 'Parent')); % calls cursor to choose set of points as seed
y = round(y); x = round(x);
outMask = ccOut == ccOut(x,y); % selects only that connected component
subplot(3,4,7), imagesc(outMask), axis image, colormap(gray), title('Final Outer Mask'); % displays closed image

% Connected component analysis to obtain inner mask
ccIn = bwlabel(closedIn, 4); % performs connect component analysis and yields labeled image
subplot(3,4,8), imagesc(ccIn), axis image, colormap(gray), title('Labeled + Closed Inner Mask'); %displays closed image
fprintf('Please click anywhere on the connected component representing the inner mask. Press enter when you are done.
'); %displays instructions
[y,x] = getpts(get(imshow(ccIn), 'Parent')); %calls cursor to choose set of points as seed
y=round(y); x=round(x);
closedIn = ccIn == ccIn(x,y); %selects only that connected component
%displays closed image

fprintf('
Please click anywhere on the connected component representing the inner mask. Press enter when you are done.
'); %displays instructions
[y,x] = getpts(get(imshow(ccOut), 'Parent')); %calls cursor to choose set of points as seed
y=round(y); x=round(x);
inMask = ccOut == ccOut(x,y); %selects only that connected component
%displays closed image

mask = ((outMask - inMask) > 0); %creates segmentation mask
mask = uint8(mask); %converts mask to type 8 unsigned integers
segIm(:,:,1) = mask.*croppedIm(:,:,1); %applies mask to each RGB component individually
segIm(:,:,2) = mask.*croppedIm(:,:,2);
segIm(:,:,3) = mask.*croppedIm(:,:,3);

subplot(3,4,11), imagesc(segIm), axis image, colormap(gray), title('Segmented'); %displays segmented blood vessel
subplot(3,4,12), imagesc(croppedIm), axis image, colormap(gray), title('Original'); %displays original for comparison

%Part 6. Calculation of Properties of Segmented Blood Vessel
OM = regionprops(outMask, 'perimeter', 'area'); %computes diameter and area of outer mask
IM = regionprops(inMask, 'perimeter', 'area'); %computes diameter and area of inner mask
stats = struct('OuterCirc', OM.Perimeter, 'InnerCirc', IM.Perimeter, 'WallCSA', (OM.Area - IM.Area)); %sets results into a struct for output
end