6-14-2023

The Effect of Post-Injury Exercise Training on Skeletal Muscle Mitochondria

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By

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A Thesis

Submitted to the Faculty of Graduate Studies

through the Department of Kinesiology

in Partial Fulfillment of the Requirements for

the Degree of Master Human Kinetics at the

University of Windsor

Windsor, Ontario, Canada

2023

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ABSTRACT

Skeletal muscle exists on a continuum of pure and hybrid fibres (I ↔ I/IIa ↔ IIa ↔ IIa/IIx ↔ IIx ↔ IIx/IIb ↔IIb) which can change phenotypes via external stimuli such as chronic exercise training. The various fibre types differ across characteristics such as shortening velocity, fatigue resistance, and mitochondrial content that provide skeletal muscle the ability to meet different metabolic, physiological, and structural demands. Another feature of skeletal muscle is its innate ability to regenerate after injury, which is a period of fibre type malleability and when fibres may be more responsive to treatments that switch phenotypes. Mitochondrial content and oxidative capacity are secondary indicators of fibre phenotype and may be similarly affected by external stimuli during exercise and regeneration due their overlapping regulatory pathways. Previous research observed an increase in fast twitch IIa/IIx hybrid fibres in the mouse soleus muscle following injury in response to endurance exercise training. Thus, this study aimed to elucidate the effect of exercise and regeneration on mitochondrial content and oxidative capacity on these hybrid fibres in the soleus. Twelve-week-old C57BL/6J mice participated in two weeks of endurance treadmill running. Fibre type-specific SDH (succinate dehydrogenase) content of type I, IIa, IIx and IIa/IIx fibres was compared between activity groups (sedentary vs exercise trained) at 14 and 50 days post-cardiotoxin-induced injury. Interestingly, 14 days of endurance exercise after an acute injury was detrimental to mitochondrial content and oxidative capacity, especially in the type IIa and IIa/IIx fibres. After 36 days of sedentary behaviour, the injured exercised group's SDH content increased to a level comparable to the control group. Thus, it appears that injury and exercise may delay the mitochondrial regenerative process and impair the early restoration of skeletal muscle oxidative capacity and fibre phenotype.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP - Adenosine Monophosphate

AMPK - AMP-activated Protein Kinase

ATP - Adenosine Triphosphate

ATP:AMP – Ratio of ATP to AMP

CaMK - Calcium/calmodulin-dependent Protein Kinase

Ca^{2+} - Calcium

ECM - Extracellular Matrix

eMHC - Embryonic Myosin Heavy Chain

ERRα - Estrogen Related Receptor Alpha

Ex – Exercised

Ex CTX – Exercised Injured

GPS – Gastrocnemius-Plantaris-Soleus Complex

HIF-2α - Hypoxia Inducible Factor 2 Alpha

HIIT - High Intensity Interval Training

IL-1β - Interleukin 1 Beta

LC3 - Microtubule Associated Proteins 1A/1B Light Chain 3A

MHC - Myosin Heavy Chain

mtDNA - Mitochondrial DNA

mTOR - Mechanistic Target of Rapamycin

mtTFA - Mitochondrial Transcription Factor A

Myf5 – Myogenic Factor 5

MyoD – Myogenic Differentiation Factor

m/min – metres per minute

NAD - Nicotinamide Adenine Dinucleotide

neoMHC - Neonatal Myosin Heavy Chain

NFAT - Nuclear Factor of Activated T Cell

Nrf1/2 - Nuclear Respiratory Factor 1 and 2

OCT – Optimal Cutting Temperature

PBS – Phosphate Buffered Saline

PGC1α - Peroxisome Proliferator-Activated Receptor Gamma

PINK1 - PTEN-induced 1

ROS - Reactive Oxygen Species

SDH – Succinate Dehydrogenase

Sed – Sedentary Control

Sed CTX – Sedentary Injured

Tfam - Mitochondrial Transcription Factor A

TGF-β - Transforming Growth Factor Beta

TNFα - Tumor Necrosis Factor Alpha

Ulk1 - Unc51-like Autophagy Activating Kinase

VDAC1 - Voltage-dependent Anion Channel 1

VEGF - Vascular Endothelial Growth Factor

WGA – Wheat Germ Agglutinin

1. REVIEW OF LITERATURE

1.1 Introduction

Skeletal muscle exists on a continuum of pure and hybrid fibres (I ↔ I/IIa ↔ IIa ↔ IIa/IIx ↔ IIx ↔ IIx/IIb ↔ IIb). It allows for the performance of various essential functions due to differing characteristics between fibre types, such as mitochondrial content, shortening velocity, and force production. Fibre type during adulthood is generally stable unless exposed to external treatments such as hormonal alterations, neural/electrical stimulation, or chronic exercise. Endurance exercise is a known contributor in fibre phenotype shifts from fast type II MHC muscle fibres toward slow type I MHC muscle fibres. When injured, skeletal muscle possesses the innate ability to regenerate which is regulated partially by mitochondrial content and turnover. These regenerative periods present unique situations where the skeletal muscle may be more responsive to external stimuli and thus more malleable to experience phenotype transitions. Additionally, the pathways that regulate fibre phenotypes overlap with those that affect mitochondrial turnover, yet the interplay between regeneration, exercise and mitochondrial turnover is largely unknown. Therefore, this study will explore the effect of exercise throughout regeneration on mitochondrial content in hybrid muscle fibres.

1.2 Skeletal Muscle and Post-injury Regeneration

Mammalian skeletal muscle is a highly malleable organ that accounts for approximately 40% of body mass in healthy adult humans (Frontera & Ochala, 2015). Skeletal muscle performs the physiological functions of locomotion, postural support, thermogenesis, pulmonary ventilation, macronutrient metabolism, and endocrine function (Schiaffino, 2010; Spriet, 2014). Given the essential nature of these functions, it is paramount to maintain skeletal muscle health for overall human health. Skeletal muscle consists of multinucleated myofibres and is

maintained, in part, by muscle stem cells referred to as satellite cells (Mauro, 1961). These stem cells remain quiescent until the muscle needs to repair or regenerate and provides it with the ability to repair damage and adapt to exercise training stimuli (Bischoff, 1990; Rosenblatt et al., 1994).

Muscle injuries, in the form of strains, blunt force trauma, eccentric contractions or toxins, present situations in which skeletal muscle must respond to restore functionality. The response to muscle tissue damage occurs in three interrelated stages: (1) inflammation, characterized by increased infiltration of neutrophils and macrophages, (2) the activation, proliferation, and differentiation of satellite cells and (3) myofibre maturation with muscle and extracellular matrix remodelling (Järvinen et al., 2013). The highly orchestrated interaction between inflammatory cells, satellite cells, and the muscle niche coordinates effective regeneration but may deteriorate from aging or disease states (Schmidt et al., 2019).

Following necrosis, neutrophil infiltration at the damage site initiates an inflammatory response. Neutrophils destroy damaged tissue through phagocytosis and the release of cytotoxins and pro-inflammatory cytokines (Toumi, 2006; Tidball, 2005). Subsequently, the first wave of macrophages (M1 macrophages) will secrete pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β) to attract immune cells and phagocytose debris as neutrophil numbers decrease (Ciciliot & Schiaffino, 2010). After 1-3 days, anti-inflammatory (M2) macrophages appear and replace M1 macrophages. Their appearance marks the beginning of tissue restoration by their secretion of transforming growth factor-β (TGF-β) (Chazaud et al., 2009). Further supporting repair, macrophages stimulate myogenic cell growth by releasing mitogenic factors. Inflammatory M1 macrophages stimulate myoblast proliferation

and inhibit differentiation, while anti-inflammatory M2 macrophages stimulate differentiation and increase myoblast fusion (Chazaud et al., 2009).

The repair phase of regeneration is characterized by the activation, proliferation, and differentiation of satellite cells. Satellite cells reside between the basement membrane and sarcolemma surrounding the myofibre in a quiescent state awaiting activation (Yin et al., 2013). Signals from the damaged environment activate quiescent satellite cells and initiate their rapid proliferation (Souza & Gottfried, 2013; Karalaki et al., 2013). Upon activation, two populations of satellite cells emerge. Satellite myogenic cells, which express MyoD, Myf5 and myogenin, differentiate into myoblasts to fuse to damaged fibres or to one another to form myofibres (Karalaki et al., 2009; Järvinen et al., 2013; Yin et al., 2013). Satellite stem cells can divide asymmetrically into myoblasts for muscle repair and stem satellite cells to replenish the quiescent satellite cell pool, or symmetrically into stem satellite cells (Kuang et al., 2007; Järvinen et al., 2013; Yin et al., 2013). The newly formed myogenic cells' fusion with existing myofibres and incorporation of new myotubes re-establishes the tissue's functionality.

The final phase of regeneration is remodelling of the damaged myofibre and extracellular matrix (ECM). Upon muscle injury, local fibroblasts increase secretion of ECM proteins such as collagens, fibronectin and laminin which contributes to the development of scar tissue in the muscle fibre (Mann et al., 2011). In early stages of injury, this provides structural stability and strength to the injured muscle fibres and will eventually allow for the re-establishment of contractile function. In instances of exacerbated inflammation, excessive ECM accumulation can impair regeneration and contribute to fibrosis (Lehto, Järvinen & Nelimarkka, 1986; Mann et al., 2011). After the remodelling phase, regenerated muscle tissue is functionally and morphologically indistinct from the uninjured muscle tissue (Karalaki et al., 2009).

1.3 Skeletal Muscle Fibre Types

Classification of Skeletal Muscle Fibre Types

The hallmark feature of skeletal muscle is its ability to drive locomotion via contraction. The diversity in contractile properties and metabolism between fibre type provides skeletal muscle with a heterogeneity that allow it to fulfill a vast range of functions (Blaauw et al., 2013). The motor unit, (i.e., a motor neuron and all the muscle fibres that it innervates) is one contributor to skeletal muscles broad functionality (Devanandan et al., 1964). Depending on the task demand, selective recruitment of the appropriate number and size of motor units allow for both fine and gross motor skills (Henneman et al., 1974). Hence, the same muscle group may contribute to high force producing movements, postural maintenance, and repeated bouts of low intensity actions (Schiaffino and Reggiani, 2011). Furthermore, motor units can be distinguished into fast or slow twitch depending on their muscle fibre type. This distinction was initially made through the observation of slow red muscle fibres rich in myoglobin and oxidative enzymes as opposed to fast white muscle fibres specializing in glycolytic metabolism (Needham, 1926). Since the initial distinctions, fibre type classification has grown immensely through various modalities for differentiation of types. Histochemical and physiological studies, electron microscopy, myosin ATPase histochemistry and biochemical enzyme studies have revealed the four predominant adult muscle fibre types accepted today (Brooke & Kaiser, 1971; Schiaffino et al., 1970; Peter et al., 1972).

The most widely accepted classification for fibre type is based on myosin heavy chain (MHC) isoform expression. With myosin ATPase histochemistry and the methods mentioned above, pure fibres, typed as I, IIa, IIb and IIx, have been defined with respect to fatigue resistance, enzymes, shortening velocity and twitch properties (Burke et al., 1970; Peter et al.,

1972; Schiaffino et al., 1989). While these four fibre types are found in mice, rabbits and rats, type IIb fibres are not found in humans (Gorza, 1990; Smerdu et al., 1994). Type I fibres are fatigue resistant via their efficient utilization of adenosine triphosphate (ATP) and high rate of mitochondrial oxidative ATP regeneration, but also possess slower shortening velocity (i.e., contraction and half-relaxation time) compared to type II fibres (Bottinelli, Schiaffino & Reggiani, 1991; Pellegrino et al., 2003). These properties make type I fibres ideal for prolonged aerobic activities. All type II fibres share similar twitch contraction force production properties yet differ in fatigue resistance and shortening velocity. Of the subgroup, type IIa are the most fatigue resistant with the slowest shortening velocity, while type IIb are least fatigue resistant with the fastest shortening velocity (Larsson et al., 1991). Type IIx measure as an intermediary between type IIa and IIb for both fatigue resistance and shortening velocity (Larsson et al., 1991; Bottinelli et al., 1994). Additionally, a plethora of mitochondrial properties within the muscle allow for characterization between fibre types. Oxidative fibres contain a greater volume of elongated mitochondria, enhanced respiratory chain activity and a highly developed mitochondrial network (Schiaffino et al., 1970; Mishra et al., 2015). As well, oxidative fibres express greater mitochondrial fusion rates linking mitochondrial dynamics with muscle fibre phenotype (Mishra et al., 2015).

Skeletal Muscle Hybrid Fibres

Type I, IIa, IIx and IIb are the four predominant fibre types expressed in adult mammalian skeletal muscle, but immunohistochemical and in situ hybridization analyses, and biochemical and physiological studies have confirmed a spectrum of pure and hybrid fibre types (Schiaffino & Reggiani, 2011). Hybrid fibres are muscle fibres that co-express two or more MHC isoforms and are commonly found in normal muscle although with wide variability across

muscle groups, intra-muscle location, and species (Medler, 2019). With the addition of hybrid fibres as intermediaries between the four most common pure fibre types, the following continuum is generated: I ↔ I/IIa ↔ IIa ↔ IIa/IIx ↔ IIx ↔ IIx/IIb ↔ IIb.

As hybrids express multiple MHC isoforms, it is hypothesized their potential to perform a wider range of functional contraction types is greater than their pure counterparts. For example, the extraocular muscles possess a sizable proportion of hybrid fibres and can perform fast twitch as well as slow tonic contractions, while a pure type IIb fibre in the vastus lateralis will only perform fast twitch contractions (Bicer & Reiser, 2009; Porter, 2002). This functional specialization provides a simple explanation to hybrid fibre abundance but is anecdotal requiring future study for a greater understanding.

Skeletal Muscle Fibre Transitions

Treatments such as chronic exercise, neural/electrical stimulation, and hormonal alterations can induce muscle fibre type switching in which hybrid fibres perform a significant role (Blaauw et al., 2013). Fibre type transitions occur in a limited capacity, as they are restricted by an "adaptive range" that is dependent on the muscle's initial fibre type, subject species, treatment duration and number of treatments (Ausoni et al., 1990; Kang & Hoh, 2010; Klitgaard et al., 1990). A fast muscle fibre will mostly transition to other fast fibres in the range of IIa↔IIx↔IIb, while slow fibres will transition between I↔IIa↔IIx (Ausoni et al., 1990). Additionally, the treatment duration may determine whether an adaptation will occur, with prolonged exposure leading to greater adaptations. This is drastically exemplified in elite level endurance runners in which 80-90% of MHC expression is type I although genetic predisposition also contributes to overall MHC expression (Baldwin & Haddad, 2001). Furthermore, the

additive effect of multiple conditions, such as a hormonal alteration combined with mechanical unloading can elicit greater fibre type transitions (Caiozzo, Baker, & Baldwin, 1998).

Hybrid fibres are frequently observed during periods of muscle fibre phenotype transition. They may serve a transient role during transitional states for muscle fibres to become pure fibres depending on environmental influences (Klitgaard et al., 1990; Medler, 2019). During early mammalian development, embryonic MHC (eMHC) is expressed, followed by neonatal MHC (neoMHC) which is then replaced by adult MHC isoforms (Adams et al., 1999; Butler-Browne et al., 1984). The transition from neonatal to adult MHC isoforms is a period in which combinations of the two MHC are expressed in individual fibres. Additionally, upon neoMHC disappearance, large proportions of hybrid fibres can persist in developing muscle of mice during maturation while some fibres transition to type IIx and type IIb (Brummer et al., 2013; Di Maso et al., 2000). Their notable presence in development emphasizes their transient role which allows for broad variability of fibre type specification depending on species, timing, and muscle group.

After embryonic development, periods of chronic exercise may also induce muscle phenotype transitions where hybrid fibres serve a transient role in the generation of pure fibres. Long distance aerobic training generally results in a reduction of hybrid fibres with a shift toward type I fibres, while resistance training interventions result in an increase in type IIa fibres and reduction in hybrid fibres (Trappe et al., 2006; Williamson et al., 2001). Though fibre phenotype transitions via exercise are generally consistent they may be altered by treatment before training commencement. For example, endurance training immediately following injury increased type IIa/IIx hybrid fibres in slow twitch muscle in mice, rather than the anticipated type I increase (Hian-Cheong, unpublished observations, 2020). Furthermore, exercise training volume may influence muscle fibre transitions as greater volume results in a decrease in type

IIa/IIx hybrid fibres in trained and untrained (Kohn et al., 2007). Additionally, studies that observe no changes in hybrid fibre proportion may indicate there is a volume threshold that must be met for adaptation to occur, agreeing with the results of Kohn et al., which demonstrated less hybrid fibres with an increased exercise volume (Medler, 2019).

1.4 Pathways Regulating Fibre Phenotype

Calcineurin-NFAT Signalling Pathway

The nerve activated, calcineurin-nuclear factor of activated T cell (NFAT) signalling pathway and AMP-activated protein kinase (AMPK) energy sensor regulate skeletal muscle fibre phenotype changes from endurance exercise (Blaauw et al., 2013; Jäger, 2007; Schiaffino et al., 2010; Olesen, 2010). The calcineurin-NFAT signalling pathway is activated through Ca^{2+}/calmodulin binding, which dephosphorylates NFAT transcriptional factors allowing for their translocation from the cytosol to nucleus and influences muscle gene programming (Schiaffino, 2010). Specifically, chronic nerve activity induces the nuclear translocation of NFATc1 causing upregulation of slow MHC expression while inhibiting fast MHC expression (Calabria et al., 2009; McCullagh et al., 2004). Overexpression and inhibition studies further corroborate the importance of calcineurin-NFAT signalling in fibre type specification. Its inhibition through the administration of cyclosporin A or other pharmacological inhibitors decreases slow MHC expression and increases fast MHC expression (Naya et al., 2000; Bigard et al., 2000; Miyazaki et al., 2006). Additionally, its overexpression in transgenic mice induces a muscle specific fibre type adaptation. Slow muscles expressed more type I MHC at the expense of type IIa MHC, and fast muscles expressed more type I, IIa and IIx MHC at the expense of IIb MHC (Talmadge et al., 2004). Since calcineurin is activated via increased cytosolic calcium and

calmodulin binding, it is also activated via increased nerve activity during endurance exercise (Qaisar et al., 2016; Wu et al., 2000).

AMPK Energy Sensor

AMPK activation occurs during muscular contractions via the increased AMP:ATP ratio (Hancock et al., 2006). Once activated, AMPK strongly interacts with the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which has an imperative role in mitochondrial biogenesis and muscle fibre phenotype (Blaauw et al., 2013; Li et al., 2002; Kjøbsted et al., 2018). The phosphorylation of PGC1α upon the activation of AMPK induces PGC1α/ERRα (estrogen related receptor-alpha) binding, and the subsequent transcription of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-2α (HIF-2α), which contributes to angiogenesis, increased mitochondrial biogenesis, and the enhancement of a slow gene program expression (Li et al., 2002; Rasbach et al., 2010; Arany et al., 2008; Cantó et al., 2009; Olesen et al., 2010). Additionally, AMPK indirectly induces PGC1α deacetylation and subsequent PGC1α/ERRα binding by increasing sirtuin1 (SIRT1) activity via increased nicotinamide adenine dinucleotide (NAD) levels (Kjøbsted et al., 2018). Since AMPK is activated by the increased ratio of AMP:ATP and PGC1α activity is upregulated by AMPK activation, this primes endurance exercise to induce slow fibre phenotype development in skeletal muscle (Olesen et al., 2010).

1.5 Skeletal Muscle Detraining

An innate characteristic of skeletal muscle is its ability to adapt to chronic treatment such as exercise training resulting in various positive outcomes such as enhanced oxidative capacity, muscle capillarization and exercise specific MHC shifts (Mujika & Padilla, 2001). Antithetically, the plasticity of skeletal muscle also allows for muscle detraining which is the reversal of these

training-induced adaptations if exercise intensity decreases or exercise cessation occurs (Booth, 1977; Coyle et al., 1984; Wibom et al., 1992; Granata et al., 2016). In only 2-3 weeks, detraining rapidly decreases oxidative enzymes to levels lower than at the point of exercise cessation before gradually reaching a new steady state (Booth, 1977; Coyle et al., 1984). This steady state is still 40-50% higher than sedentary controls and can last for up to 12 weeks, therefore training-induced adaptations are not immediately and completely abolished (Chi et al., 1983; Coyle et al., 1984). As well, decreases in mitochondrial enzyme activity coincide with a decline in ATP production, although detrained ATP production remains greater than pretraining levels (Wibom et al., 1992). Inactive periods may be involuntary or voluntary caused by injury, surgery, and illness or simply periods of rest after intense training periods/seasons (Neufer, 1989). Regardless of reason, it is of interest to maintain the health benefits associated with exercise training during periods of brief inactivity. Therefore, examining treatments that may reduce or delay the loss of training-induced adaptations has practical application as the consistent maintenance of a training program is not realistic.

1.6 Mitochondrial Turnover and Exercise

Mitochondrial Biogenesis and Exercise

Colloquially termed “the powerhouse of the cell,” mitochondria are organelles that are central in many processes involved in cellular metabolism including ATP generation, β-oxidation of fatty acids, Krebs cycle and calcium handling (Mishra & Chan, 2016; Memme et al, 2021). Mitochondrial homeostasis is paramount in the maintenance of organismal health, as imbalance and/or dysfunction are observed in neurodegenerative and metabolic diseases (Nunnari & Suomalainen, 2012). Homeostasis is achieved through mitochondrial dynamics, which is the interplay between fusion and fission, as well as biogenesis and mitophagy. These

processes allow for dysfunctional portions of the organelles to be removed, more organelles to be produced or multiple mitochondria to fuse together depending on cellular needs (Mishra & Chan, 2015).

A key regulator of skeletal muscle mitochondrial biogenesis and oxidative metabolism is PGC1α. When stimulated, it increases mitochondrial gene transcription through the induction of nuclear respiratory factor 1 and 2 (Nrf1 and Nrf2) and ERRα (Wu et al 1999; Handschin, 2006). The coactivation of these genes by PGC1α create a positive feedback loop in which Nrf2 and ERRα promote their own, each other's, and Nrf1's gene expression (Mootha, 2004). Subsequently, Nrf1/2 induce the transport of mitochondrial transcription factor A (Tfam) into the organelle, where it upregulates the expression of mtDNA-derived proteins (Gordon et al., 2001).

Endurance training and high intensity interval training (HIIT) are potent modalities which upregulate mitochondrial biogenesis, but the frequency, duration, and intensity of exercise must be sufficient to drive mitochondrial adaptations (MacInnis et al, 2017; Perry et al, 2010; Holloszy, 1967). Additionally, mitochondrial adaptation can occur in any fibre type, but the magnitude of change is dependent upon initial mitochondrial content (Lundby & Jacobs, 2016). In humans, mitochondrial content is greatest in type I fibres, but type IIa fibres elicit the greatest mitochondrial adaptations via exercise, regardless of modality (i.e., HIIT or endurance) (Howald et al., 1985; Lundby & Jacobs, 2016). The initiation of acute exercise activates multiple signalling pathways which converge on PGC1α and upregulate mitochondrial biogenesis which include: (1) the nerve-mediated calcium/calmodulin-dependent protein kinase (CaMK) pathway (Ojuka et al., 2003), (2) activation of p38 mitogen activated protein kinase (p38 MAPK) (Akimoto et al., 2005) and (3) AMPK phosphorylation (Winder et al., 2000).

Mitochondrial Autophagy and Exercise

Though exercise stimulates pathways that promote mitochondrial biogenesis, the degradation and clearance of dysfunctional organelles is paramount to maintain mitochondrial health in the muscle (Memme et al., 2021). Organelles that possess a decreased membrane potential and/or excessive production of reactive oxygen species (ROS) are flagged for degradation and subsequent mitophagy via the PTEN-induced 1 (PINK1) and Parkin pathway (Mishra & Chan, 2016; Narendra et al., 2010). Under normal conditions, PINK1 is constantly transported into the mitochondria and degraded by matrix processing-peptidases (MPP) (Matsuda et al., 2010). When mitochondrial membrane potential is lost, PINK1 no longer transports into the mitochondria and accumulates on the outer membrane (Matsuda et al., 2010; Narendra et al., 2010). Its stabilization facilitates the recruitment of Parkin to the mitochondria which mediates the ubiquitination of outer-membrane proteins such as mitofusin 1/2 and voltage-dependent anion channel 1 (VDAC1), tagging the organelle for degradation (Geiser et al., 2010; Tanaka et al., 2011; Narendra et al., 2008).

Situations of cellular stress, such as exercise, can stimulate the PINK1/Parkin pathway and upregulate mitophagy through the activation of AMPK and localization of Parkin to the mitochondria (Egan et al., 2011; Chen et al., 2018). AMPK activation induces the building of autophagic machinery via the phosphorylation of Unc51-like autophagy activating kinase 1 (Ulk1), a mediator to phagophore formation, and inhibits the growth-promoting mechanistic target of rapamycin (mTOR) pathway, which suppresses Ulk1 activity (Egan et al., 2011; Mishra & Chan, 2015; Kim et al., 2011). These processes act as the initial steps for mitophagy to occur. Additionally, PGC1α appears to play a regulatory role in the immediate mitophagy response following exercise, as its knockout results in the impairment of Parkin localization and mitophagy signalling and flux compared to controls (Vainshtein et al., 2015). Repeated bouts of

exercise, such as during endurance training, primes muscle to clear dysfunctional organelles by improving mitophagy signalling and reducing mitophagy flux (Guan et al., 2019; Lira et al., 2013; Chen et al., 2018).

1.7. Mitochondria and Skeletal Muscle Health

Oxidative Capacity and Skeletal Muscle Outcomes

Skeletal muscle oxidative capacity is a measure of the muscles' ability to aerobically generate ATP (Russ & Kent-Braun, 2000). As physical activity improves mitochondrial content and quality, and mitochondria are the site of oxidative phosphorylation, oxidative capacity is greatly influenced by exercise (Laughlin et al., 1990). This also makes oxidative capacity imperative in the determination of skeletal muscle health as it provides insight into whether the mitochondria can support the metabolic demands of the muscle (Zampino et al., 2020). A by-product of oxidative phosphorylation are ROS which are important regulators for cellular growth, proliferation, and differentiation at low levels (Davies et al., 1982; Scicchitano et al., 2018; Sena & Chandel, 2012). Under normal circumstances, mitochondria handle ROS through the release of antioxidant enzymes, such as superoxide dismutase (SOD2) (Napalitano et al., 2021). With the assistance of catalase and glutathione, antioxidant enzymes neutralize the reactivity of ROS and reduce the risk of potential damage they may induce (Kozakowska et al., 2015; Murphy, 2009).

Oxidative stress occurs when the production of ROS is greater than the cell's ability to handle them, which can induce mitochondrial DNA mutations, damage to the mitochondrial membrane and damage to the respiratory chain (Nunnari & Suomalainen, 2012; Ademowo et al., 2017). Certain situations of cellular stress, such as hypoxia, injury or strenuous exercise can generate an exorbitant amount of ROS in the muscle. Furthermore, the combination of multiple

ROS contributors, such as strenuous exercise at high altitude, can have synergistic effects which dramatically increase the release of free radicals (Quindry & Jackson, 2016; Debevec at al., 2017). Prolonged oxidative stress is evident in various pathologies such as cancer cachexia, sarcopenia and muscular dystrophy which contributes to worsened oxidative capacity (Penna et al., 2020; Vitorino et al., 2015; Lee & Wei, 2012; Mosca et al., 2021). Therefore, the maintenance of mitochondrial health via mitochondrial biogenesis and autophagy to reduce oxidative stress is imperative in regulating skeletal muscle health.

Mitochondrial Turnover as a Regulator of Skeletal Muscle Regeneration

Muscular regeneration, mitochondrial biogenesis, and mitochondrial autophagy occur concomitantly after skeletal muscle injury, with evidence supporting biogenesis and autophagy as important regulatory events during regeneration (Duguez et al., 2002; Rochard et al., 2000; Call et al., 2017). In regenerating skeletal muscle, alteration of mitochondrial activity influences myogenin expression, an important gene involved in myogenesis. Inhibition of mitochondrial activity via chloramphenicol administration reduces myogenin expression and decreases myoblast population, while stimulation of mitochondrial activity via p43 overexpression upregulates myogenin expression (Pessemesse et al., 2019; Rochard et al., 2000). Additionally, the importance of autophagy during the early stages of regeneration is characterized by the upregulation of multiple autophagy related proteins, which include Ulk1, Beclin and microtubule-associated proteins 1A/1B light chain 3A (LC3) which maintain the expanding mitochondrial network (Call et al., 2017). Furthermore, a deficiency in Ulk1, and Ulk1-mediated mitophagy, is associated with an impairment in functional muscular regeneration and mitochondrial network recovery (Call et al., 2017). Lastly, elevated levels of PGC1α and mitochondrial transcription factor A (mtTFA) occur at the onset of muscle differentiation, and

poor muscle regeneration occurs when mitochondrial activity is inhibited, corroborating the role of mitochondrial biogenesis during muscle regeneration (Duguez et al., 2002; Wagatsuma et al., 2011).

The Effect of Exercise Training During Regeneration on Mitochondrial Development

The relationship between skeletal muscle and mitochondrial dynamics during exercise or regeneration is well established. Functional regeneration follows the three interrelated stages of inflammation, satellite cell activation/differentiation and ECM remodelling, but can be impaired if mitochondrial biogenesis and/or autophagy are disturbed (Järvinen et al., 2013; Call et al., 2017). Additionally, endurance exercise training induces a slow gene phenotype in skeletal muscle and promotes mitochondrial biogenesis with enhanced mitochondrial quality, consistent with mitochondria observed in naturally occurring type I fibres (Calabria et al., 2009; Guan et al., 2019). Although, results from Hian-Cheong (2020) contrasts the slow gene fibre phenotype shift typically observed during endurance exercise training as cardiotoxin injury prior to an endurance exercise protocol induced an increase in type IIa/IIx hybrid fibres in the soleus. With this knowledge, along with the overlap that exists between pathways that regulate mitochondrial turnover, skeletal muscle regeneration, and muscle fibre phenotype transitions, it is of interest to explore how endurance exercise training during regeneration influences mitochondrial content and respiratory chain activity.

This has led to the following research question: does endurance exercise throughout skeletal muscle regeneration affect skeletal muscle oxidative capacity within hybrid fibres? To answer this question, mice undergoing muscle regeneration will be exercise-trained and have those muscles subjected to histological analyses to determine mitochondrial levels across regenerating pure and hybrid fibres.

2. METHODS

2.1 Animal Handling and Procedures

Animal Housing and Muscle Injury

Sixteen male C57BL/6J mice purchased from the Jackson Lab (Bar Harbor, ME) received a cardiotoxin (CTX) injection injury into their left leg and were randomly assigned into an exercise treatment or sedentary group. The right leg would serve as the uninjured group in the sedentary (Sed) and exercise (Ex) groups while the left leg would serve as the injured group in the sedentary (Sed CTX) and exercise (Ex CTX) groups. Furthermore, all groups were subdivided randomly into a 14- or 50-day post-CTX injury time point group (n=4). Only male mice were selected to eliminate the potential influence of fluctuating hormones and growth factors observed during the estrous cycle. The mice were housed in a 22°C animal room following a 12:12h light-dark cycle at 50% humidity. Water, a standard diet *ad libitum*, and nesting material along with cardboard tubing for enrichment were provided. Acclimatization to housing occurred one week prior to handling.

Forty-eight hours prior to exercise commencement muscle damage was induced via a 50 µl intramuscular injection of 10 µM cardiotoxin (CTX; Latoxan, France; Fig. 1). CTX was administered in a single injection to the left TA and in three evenly spaced injections in the gastrocnemius-plantaris-soleus (GPS) complex in the injury groups. CTX is a myonecrotic agent that is commonly used to induce injury and subsequent regeneration because of its ease of use and reproducibility (Garry et al., 2016). The current study's injury model injected CTX into the left GPS of each mouse while the right leg remained unharmed. Once the exercise protocol was completed and muscle samples were collected, the left leg was termed the *injured* leg and the right leg *uninjured***.**

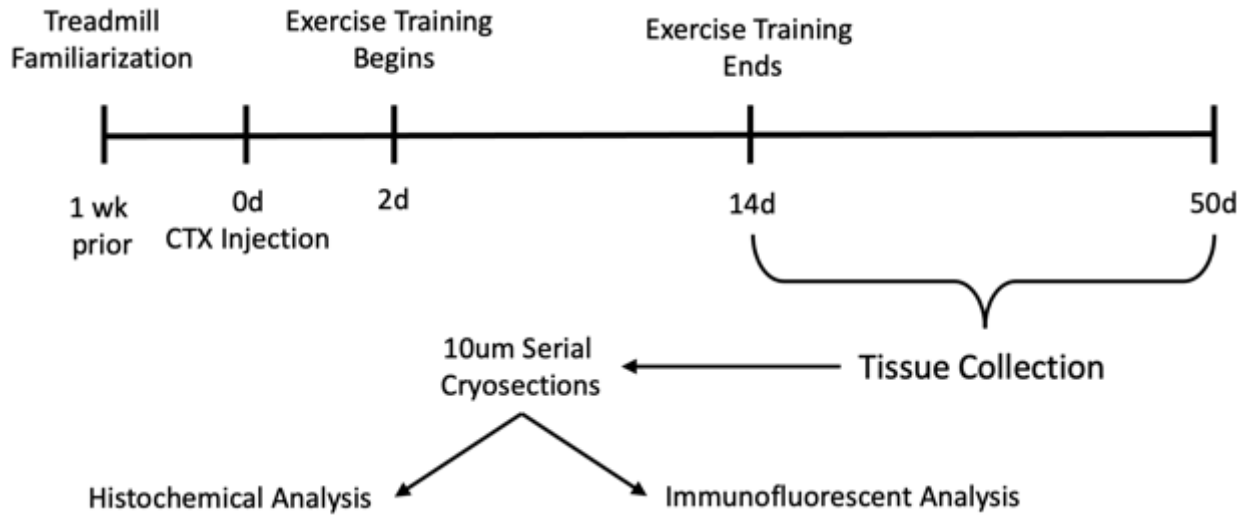

Figure 1. Experimental timeline. Treadmill familiarization period began one week prior to CTX injection. Exercise training began two days afterward. At 14 and 50 days post-injury mice were sacrificed, and muscle tissue was collected. 10 μm serial cryosections were obtained using Leica CM1860 UV cryostat and mounted on glass slides. Serial sections were stained with MHC antibody cocktail or SDH/WGA647 stain combination.

Exercise Protocol

Mice were familiarized with the treadmill for four 10-minute sessions one week prior to CTX treatment (Fig. 1). The first session acted as an introduction as the treadmill had no grade and remained stationary. The second session maintained the 0% grade but increased the speed to 5m/min. The third and fourth sessions increased the grade to 5% and increased the speed to 7.5m/min.

The endurance exercise training protocol commenced 48 hours following CTX treatment and consisted of 40-minute sessions, four sessions per week across two weeks (Fig. 1). Including a five-minute warm up before each session and five-minute cool down period after each session, the total time on the treadmill per session was 50 minutes. The first session had a grade of 10% and speed of 14m/min for the first 20 minutes and increased to a 15% grade and 15m/min speed for the remaining 20 minutes (Fig. 2). The first 20 minutes of the subsequent session increased

the speed by 1 m/min from the prior session's end speed and the remaining 20 minutes added 1m/min from the first 20 minutes (i.e., session two will begin at 16m/min for the first 20 minutes and increase to 17m/min for the remaining 20 minutes). This pattern continued until exercise training completion. The treadmill grade was always 10% for the first 20 minutes of a session and 15% for the remaining 20 minutes. To encourage the mice to continue running without the use of electric shock, flexible bristle brushes were placed at the end of each running lane. Upon contacting the brushes, the mice would increase cadence.

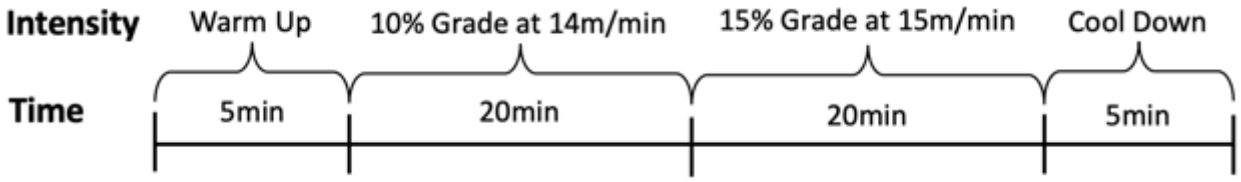

Figure 2. Baseline exercise protocol underwent by Ex and Ex CTX mice. Subsequent training sessions increased intensity by 1m/min every 20 minutes. Each session began with a five-minute warm up and ended with a five-minute cool down.

Tissue Collection

Mice were euthanized at 14 and 50 days post-CTX treatment via exsanguination followed by cervical dislocation. The soleus was harvested and plated with optimal cutting temperature (OCT) compound on a folded piece of tin foil and placed in liquid nitrogen-cooled isopentane. Frozen tissues were transferred to a 2mL Eppendorf tube and stored at -80°C.

Histological Analyses

10 µm serial cryosections of muscle tissue were obtained using a Leica CM1860 UV cryostat and mounted on VWR Superfrost Plus Micro slides (48311-703, VWR). These samples incubated in M.O.M. (Mouse on Mouse) blocking reagent (MKB-2213-1, MJS BioLynx Inc) in phosphate buffered saline (PBS) (2 drops per 2.5ml PBS) for one hour at room temperature. Samples were washed with PBS three times for five minutes and then incubated in a blocking

buffer (10% normal goat serum, 0.2% Triton X-100 in PBS) for 30 minutes at room temperature. Samples were then immunostained overnight at 4°C in blocking buffer with primary antibodies against type I (6H1) (1:50), IIa (SC-71) (1:600), IIx (BA-F8) (1:50) myosin heavy chain to identify fibre types as per previous studies (Bloemberg & Quadrilatero, 2012). Afterward, samples were PBS washed three times for five minutes before being stained with secondary antibodies goat anti-mouse IgG2b, Alexa Fluor 488 (A-21141), goat anti-mouse IgG1, Alexa Fluor 555 (A-21127), and goat anti-mouse IgM, Alexa Fluor 647 (A-21238) in PBS for one hour in the dark at room temperature. Samples were again PBS washed and stained with DAPI (1:2000 in PBS) for five minutes at room temperature. After a final PBS wash, fluoromount and a coverslip were applied. The primary antibodies were purchased from Developmental Studies Hybridoma Bank (University of Iowa) and secondary antibodies were purchased from Thermo Fisher Scientific. Images were captured on a Nikon Eclipse 90i upright epiflourescent and brightfield microscope with the 20x objective lens. The MHC stain only used the microscopes epiflourescent channels. A negative primary antibody test was done to confirm specificity and determine appropriate gain and exposure time for each channel which was consistently used across all samples for all treatments and time points.

The adjacent serial sections were histochemically stained for SDH to characterize mitochondrial content which could then be compared to the fibre type serial section. Sections were incubated at 37°C for 19 minutes in a medium containing sodium succinate dibasic hexahydrate (3.38 mg/ml) (S2378, Sigma-Aldrich), nitro blue tetrazolium chloride (0.5 mg/ml) (N6876, Sigma-Aldrich), and phenazine methosulphate (0.175 mg/ml) (P9625, Sigma-Aldrich) in distilled water and PBS, as per Blanco et al. (1988). After incubation, slides were quickly rinsed with distilled water three times for one minute before being incubated with wheat germ

agglutinin (WGA) conjugated with Alexa Fluor 647 (5µg/ml) (W32466, Thermo Fisher Scientific, Canada) in PBS for one hour and 30 minutes in the dark at room temperature. WGA is a lectin which binds to N-acetylglucosamine and N-acetylneuraminic acid residues used to label mammalian cell membranes, as per previous studies (Kostrominova, 2011). After a PBS wash (three times for five minutes) DAPI (1:2000) was applied for five minutes at room temperature. Slides were then fluoromounted and coverslipped after a final PBS wash (three times for five minutes). DAPI was used on both the MHC and SDH stained sections to locate nuclei and identify centrally nucleated myofibres to confirm regenerating regions. Only these regenerating fibres were analysed in the injured groups. Additionally, at the 14-day time point, regenerating muscle fibres were occasionally limited to the periphery of the soleus which restricted the amount of measurable regenerating fibres to measure. Images were captured on a Nikon Eclipse 90i upright epiflourescent and brightfield microscope with the 20x objective lens. The SDH stain used the epiflourescent channels (for nuclei and fibre borders) and brightfield channel (for the SDH stain). The fibres in the SDH serial sections were then compared to the MHC stain to provide values for individual fibre types (Fig. 3).

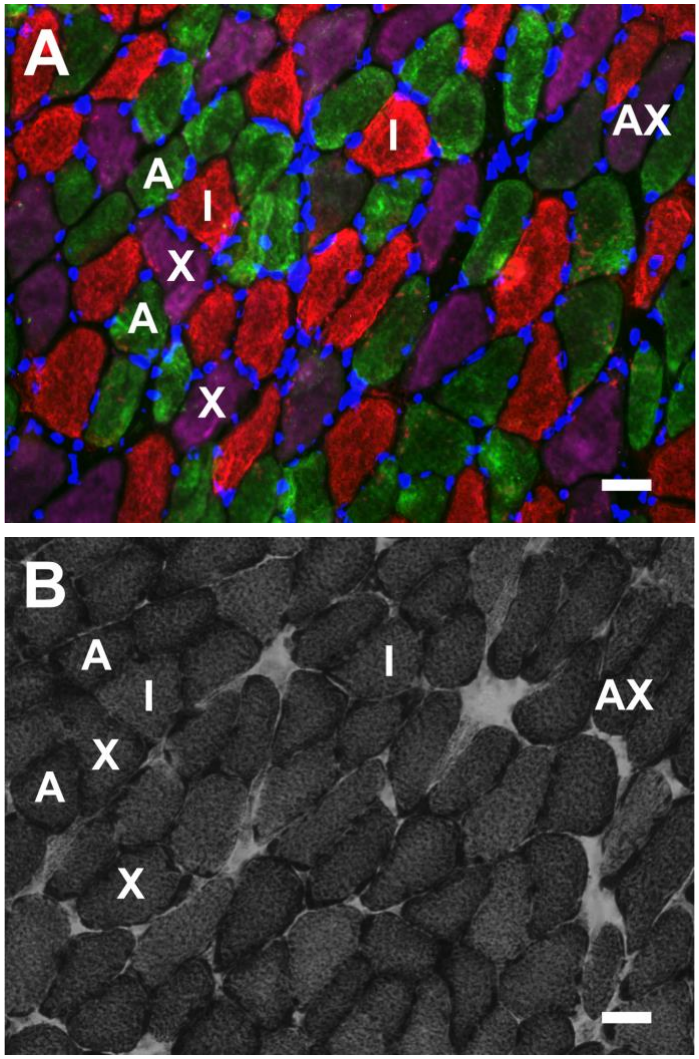

Figure 3. Immunofluorescent and histochemical staining of serial sections of exercised mouse soleus (non-injured) at the 50-day timepoint. Serial sections of mouse soleus stained with (A) MHC antibody cocktail (BA-F8, 6HI, SC-71) with DAPI and (B) SDH to serially identify fibre type and SDH activity. Examples of type I (I), IIa (A), IIx (X) and IIa/IIx (A/X) are identified in the serial images. Scale bars represent 50 μm.

Automated Fibre Segmentation

Numerous images of the WGA647 stains (Fig. 4A) were processed in the anatomical segmentation algorithm software *Cellpose* (version 2.0) to train a model that would identify muscle fibre borders based on these specific stains (Stringer et al., 2021; Waisman et al., 2021) (Fig. 4A). Once trained, WGA647 images were input into *Cellpose* which automatically generated muscle fibre masks (Fig. 4B) which were manually corrected to accurately encapsulate individual muscle fibres. Masks were then converted to segmented outlines (Fig. 4C) and input into image processing software *ImageJ*. Using *ImageJ* plugin LabelsToROI developed by

Waisman et al. (2021), the segmented outline was overlayed on the SDH stain (Fig. 4D) and eroded by one pixel to reduce intermuscular measurements. Mean gray value of each segment was calculated using the LabelsToROI plugin with a more darkly stained fibre equating to greater SDH activity.

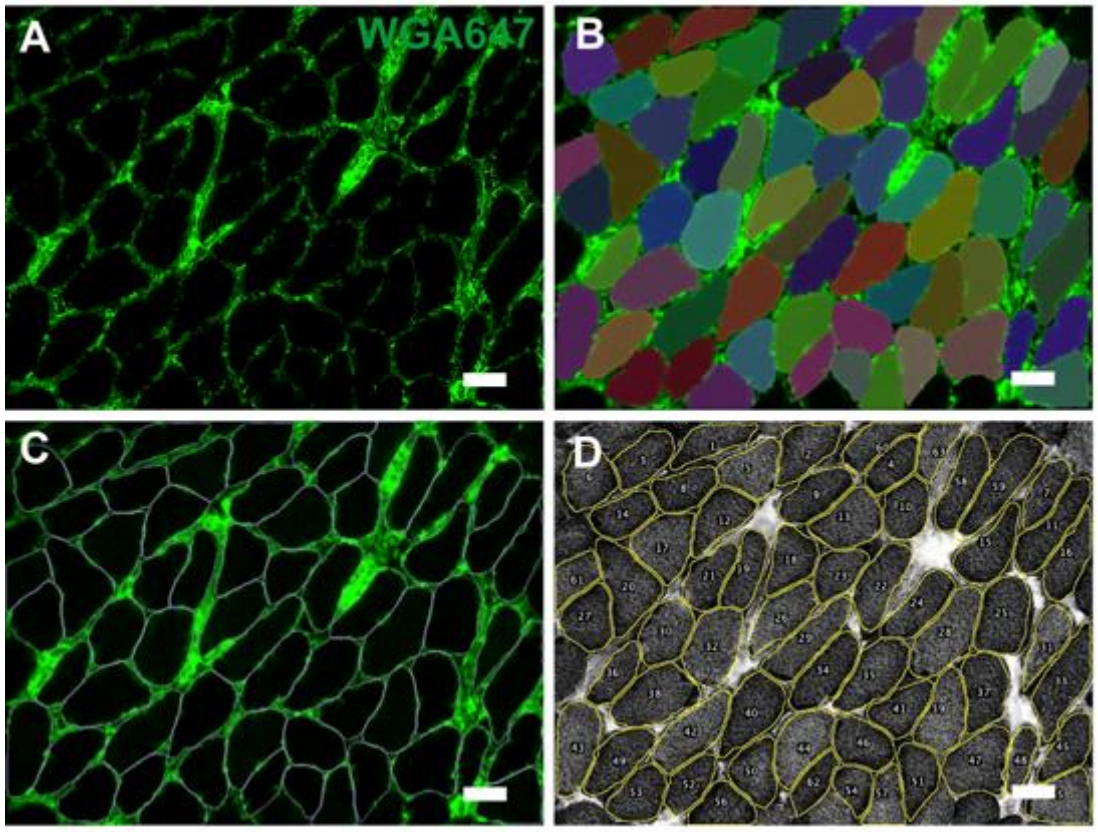

Figure 4. Images demonstrating the workflow of calculating mean gray values per muscle fibres. (A) WGA647 fluorescent stain identifying muscle fibre borders. (B) Fibre mask generated by trained *Cellpose* UI masking software. (C) The fibre mask is converted to fibre border outlines to be used in image processing software *ImageJ*. (D) Individualized mask overlayed on respective SDH stain. Borders were eroded by 1 pixel for fibre outline and mean gray value calculated within the outline. Scale bars represent 50 μm.

2.2 Data and Statistics

A three-factor ANOVA was conducted to determine the effects and interactions of the independent variables exercise, muscle injury, and time post-injury on the dependent variable mitochondrial content within each fibre type including hybrid IIa/IIx fibres measured via mean

grey value of the individual fibres. Levene's test was used to test for homogeneity of variances, Shapiro-Wilk's test for normality was used for assessment of distribution and boxplots were used to detect outliers. Mice were grouped as sedentary (Sed), exercise (Ex), sedentary injured (Sed CTX), and exercise injured (Ex CTX) at 14 and 50 days post-CTX injury. Simple main effects testing was performed to determine pairwise differences when significant interactions were observed (Bibby, 2010). Analyses were performed using JASP statistical software packages and considered significant if below an alpha level of 0.05.

3. RESULTS

Body and Muscle Mass

Body mass values were collected and analyzed during a previous study by Dylan Hian-Cheong (2020). Data from Hian-Cheong (2020) are solely for descriptive purposes. Refer to *The Effects of Exercise Following Skeletal Muscle Injury on Fibre Type Composition* for a more thorough analysis of the different muscle groups studied along with MHC phenotype transitions undergoing the same injury and exercise model. Body mass was assessed three days post-CTX injury (pre-exercise) and immediately after mice were sacrificed (post-exercise). Both the Sed and Ex groups showed a positive trend of weight gain between the pre-exercise and 50-day time point. The differences between the activity groups were not significant and there was no significant interaction between activity and time point on change in body mass (Hian-Cheong, 2020).

There was no individual soleus weight as the gastrocnemius, plantaris and soleus were excised together as the triceps surae. This allowed for the facilitation of cryosectioning, histochemical staining and immunofluorescent staining. There was no significant change in

weight over the 50 days in the uninjured GPS or between activity groups (Hian-Cheong, 2020). The injured GPS mass significantly increased over the 50-day period but did not significantly differ between activity groups. The mice studied by Hian-Cheong (2020) for fibre phenotype transitions were used in the current study examined for changes in oxidative capacity.

SDH Activity of Soleus Myofibres

The soleus is a slow twitch muscle which is recruited during endurance exercise. In mice, it is primarily composed of type I and type IIa fibres, with type I fibres typically being less oxidative than type II fibres (Crow & Kushmerick, 1982; Bloemberg & Quadrilatero, 2012). This muscle was specifically chosen because a prior study observed an increase in type IIa/IIx hybrid fibres in the soleus when injured and exercised (Hian-Cheong, 2020). The SDH stain resulted in more darkly stained fibres that were richer in active mitochondria (Fig. 5).

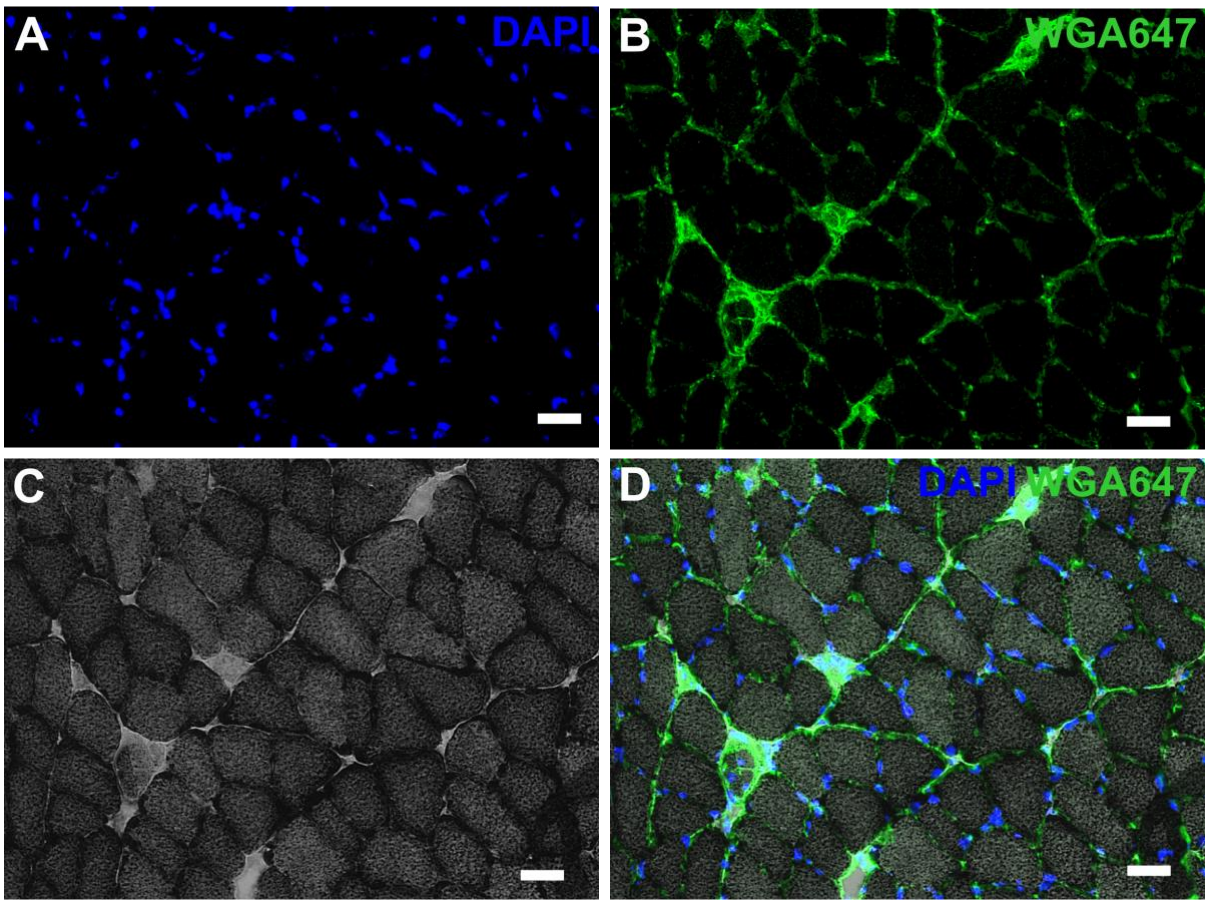

Figure 5. Fluorescent and histochemical stain of sedentary uninjured mouse soleus at 50 days post-CTX injury. (A) Nuclei (DAPI), (B) muscle fibre borders (WGA647), and (C) mitochondrial activity (SDH) were stained in a combined protocol. Darker SDH stained fibres indicate greater mitochondrial content. (D) Merged image of A-C. Scale bares represent 50 μm.

The soleus was also given an immunofluorescent stain against primary MHC antibodies to allow for measurement of fibre specific SDH activity (Fig. 6). The SDH activity of the type I, IIa, IIx and IIa/IIx fibres were measured at 14 and 50 days post-CTX injury in each group. Type I/IIa hybrid fibres were not measured as there were not enough for comparison between and within treatment groups (Fig. 6).

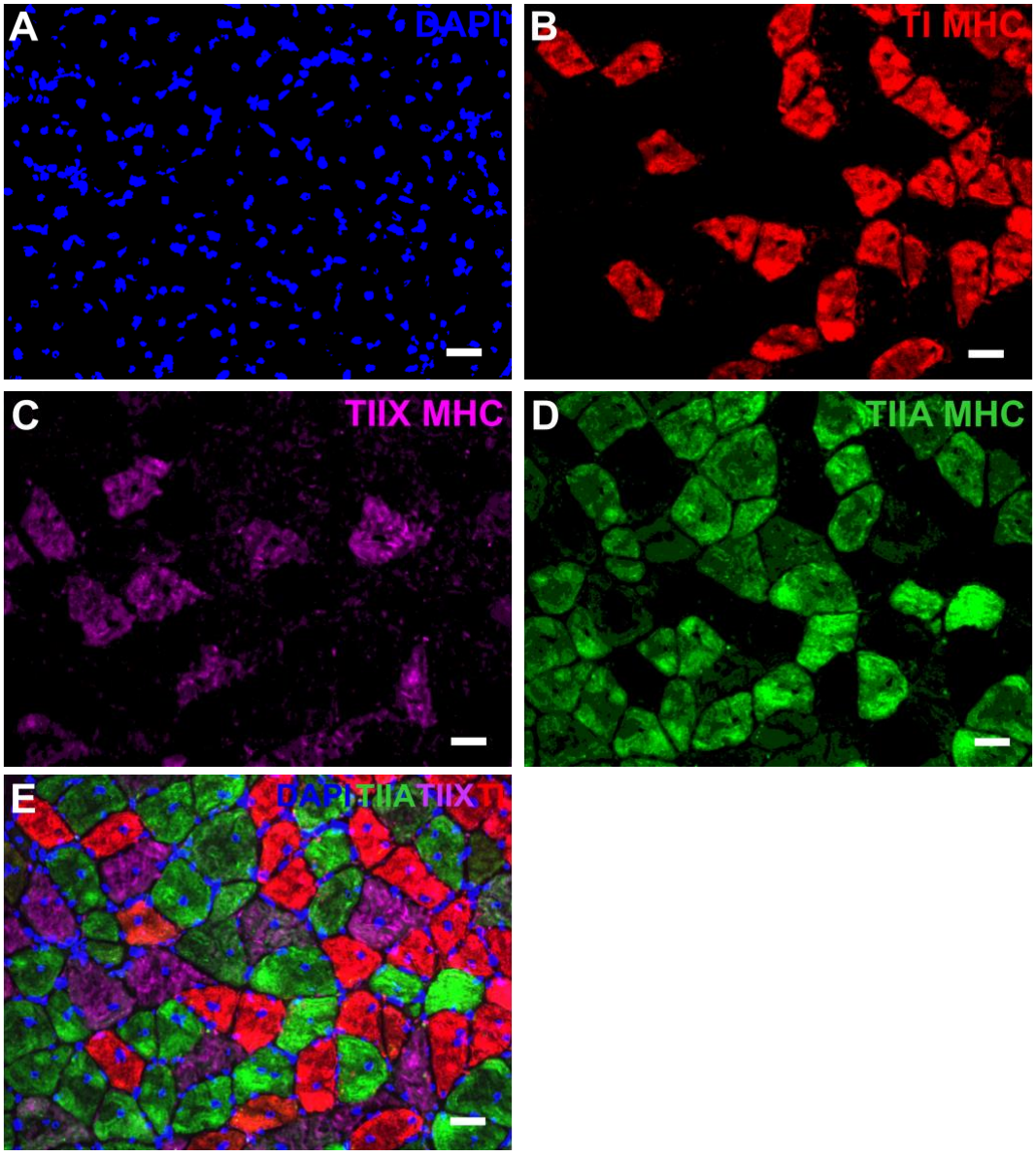

Figure 6. Immunofluorescent MHC stain of injured exercised mouse soleus at 50 days post CTX injury. (A) DAPI was used to identify nuclei to confirm regeneration via the presence of centrally nucleated muscle fibres. (B-D) Antibody cocktail staining for (B) BA-F8 (type I MHC), (C) 6H1 (type IIx MHC) and (D) SC-71 (type IIa MHC). (E) Merged channel image of myosin heavy chain antibody cocktail (B-D). Scale bars represent 50 μm.

In the type I fibres the change in SDH activity from 14 to 50 days post-CTX injury had a significant main effect of time post-CTX injury ($p<0.001$) (Fig. 7A). Similarly, the change in SDH activity for the IIx fibres showed a significant main effect for time post-CTX injury ($p<0.001$) (Fig. 7C). The change in type IIa fibre SDH activity from 14 to 50 days post-CTX injury showed a significant main effect for time post-CTX injury ($p<0.001$) and injury ($p=0.023$) (Fig. 7B). The change in SDH activity in the type IIa/IIx hybrid fibres also showed a significant main effect for time post-CTX injury ($p<0.001$) and injury ($p=0.029$) and a significant interaction between time post-CTX injury and injury ($p=0.041$) (Fig. 7D). Lastly, when SDH activity for all fibre types was combined, there was a significant main effect of time post-CTX injury ($p<0.001$) (Fig. 7E). Most importantly, there was a significant three-way interaction between time post-CTX injury, exercise, and injury in all fibre types (type IIa ($p<0.001$); type IIa/IIx ($p<0.001$); type I ($p<0.001$); type IIx ($p=.043$); combined fibre group ($p<0.001$)) which suggests that the effect of injury and exercise was hindering SDH activity at 14 days, but rebounded after 36 days of inactivity (Fig. 7A-E).

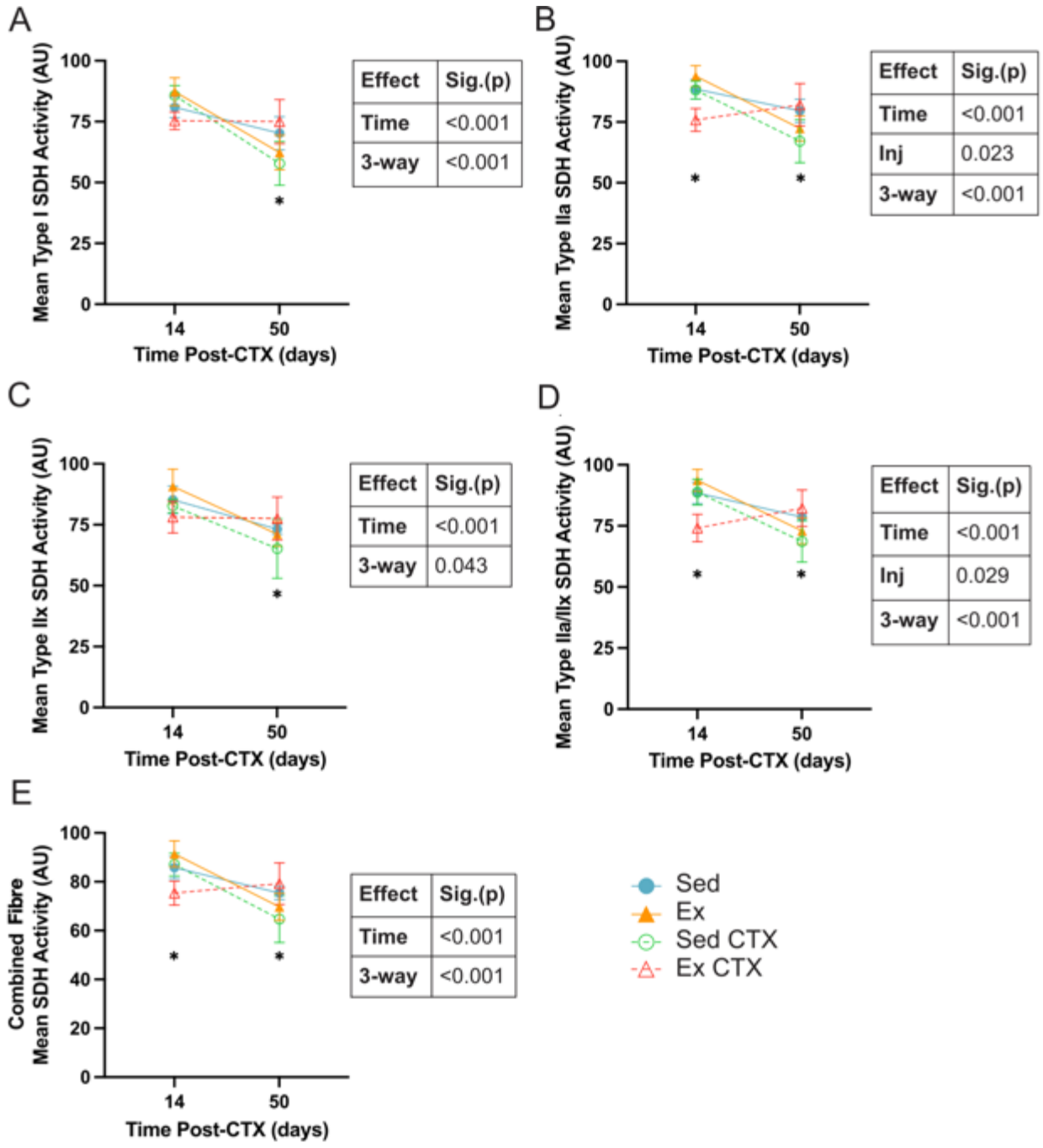

Figure 7. Histochemical analysis of mean SDH activity in injured and uninjured soleus muscle of exercise and sedentary mice at 14 and 50 days post-CTX injury. Data are presented as mean SDH activity (A-E). SDH activity in the type I, IIa, IIx, IIa/IIx, and combined fibres all demonstrated a main effect of time and three-way interaction effect of injury, time, and exercise (A, B, C, D, E). There was a simple main effect of activity in the type IIa, IIa/IIx, and combined fibres between the Sed CTX and Ex CTX groups at the 14-day timepoint (B, D). From the 14-day to 50-day timepoint, in the type I, IIa, IIx and IIa/IIx fibres, the Ex CTX SDH activity increased while the Sed CTX SDH activity decreased resulting in a simple main effect of activity in all fibre types at 50 days (A, B, C, D, E).
* = *simple main effect of activity between Sed CTX and Ex CTX at given time point*

4. DISCUSSION

Skeletal muscle exists on a continuum of pure and hybrid fibres (I↔I/IIa↔IIa↔IIa/x↔IIx↔IIx/b↔IIb) which enables it to meet various physiological, metabolic, and structural demands. During adulthood, myofibre phenotypes typically remain unchanged, but treatments such as chronic neural/electrical stimulation or endurance training can induce fast twitch (type II) to slow twitch (type I) phenotype transitions (Blaauw et al., 2013). During these transitions hybrid muscle fibres are thought to act as intermediaries for type IIx fibres transitioning to type IIa fibres, or type IIa fibres transitioning to type I fibres resulting in an increase in the pure to hybrid fibre ratio (Medler, 2019). An innate characteristic of skeletal muscle is its ability to regenerate after trauma. Regeneration marks a period in which skeletal muscle undergoes a series of fibre type transitions from IIb to IIx to IIa suggesting that it is more malleable and may be susceptible to external stimuli to increase fibre type transitions (Launay et al., 2006; Ausoni et al., 1990; Matsuura et al., 2007; Dalle et al., 2020). Mitochondrial content and oxidative capacity act as secondary indicators of muscle fibre phenotypes and may be affected similarly by external stimuli during regeneration. As the pathways that regulate oxidative capacity and mitochondrial content overlap with those that influence fibre type transitions via exercise, it was the aim of this study to examine the effect of injury followed by endurance training on oxidative capacity in each fibre type.

Previous studies examining fibre type transitions from chronic exercise observed an increase in pure MHC fibres at the expense of hybrid fibres regardless of training type (Trappe et al., 2006; Williamson et al., 2001). During these transitions, the hybrid muscle fibres are thought to act as intermediaries for type IIx fibres transitioning to type IIa fibres, or type IIa fibres transitioning to type I fibres (Medler, 2019). In a prior experiment by Hian-Cheong (2020), it

was found that regenerated IIa/IIx hybrids increased in the soleus muscle following injury and subsequent initiation of endurance exercise training. This result differed from past research on exercise and fibre type transitions suggesting a possible positive adaptation in the regenerated IIa/IIx hybrid fibres. Conversely, it may have represented a delay in the progression of establishing the type IIa fibre pool from IIx fibres. Therefore, it was worthwhile to examine secondary indicators of fibre type identity such as SDH as a marker of oxidative capacity.

Fourteen days post-injury, regardless of fibre type, the Ex CTX mice had the lowest SDH activity of any group (Fig. 7E). The simple main effects test showed SDH activity was significantly lower in the Ex CTX group compared to the Sed CTX group in the type IIa ($p=0.012$) and type IIa/IIx fibres ($p=0.003$) at 14 days post-injury. The significantly lower SDH activity in the Ex CTX mice indicates a decrease in mitochondrial content or mitochondrial activity compared to the other groups (Fig. 8) (Larsen et al., 2012). In healthy muscle, exercise improves mitochondrial content and quality by clearing dysfunctional organelles while increasing mitochondrial biogenesis to produce new mitochondria (Holloszy, 1967; Salminen & Vihko, 1984; Lira et al., 2013). Fourteen days after injury the mitochondrial network should be re-established, a process that typically takes 5-10 days post-injury (Call et al., 2017; Duguez et al., 2002). This is exemplified by the similar SDH activity measured in the Sed and Sed CTX groups (Fig. 7E) (Sed = 85.77 ± 4.56 AU, Sed CTX = 87.04 ± 4.62 AU).

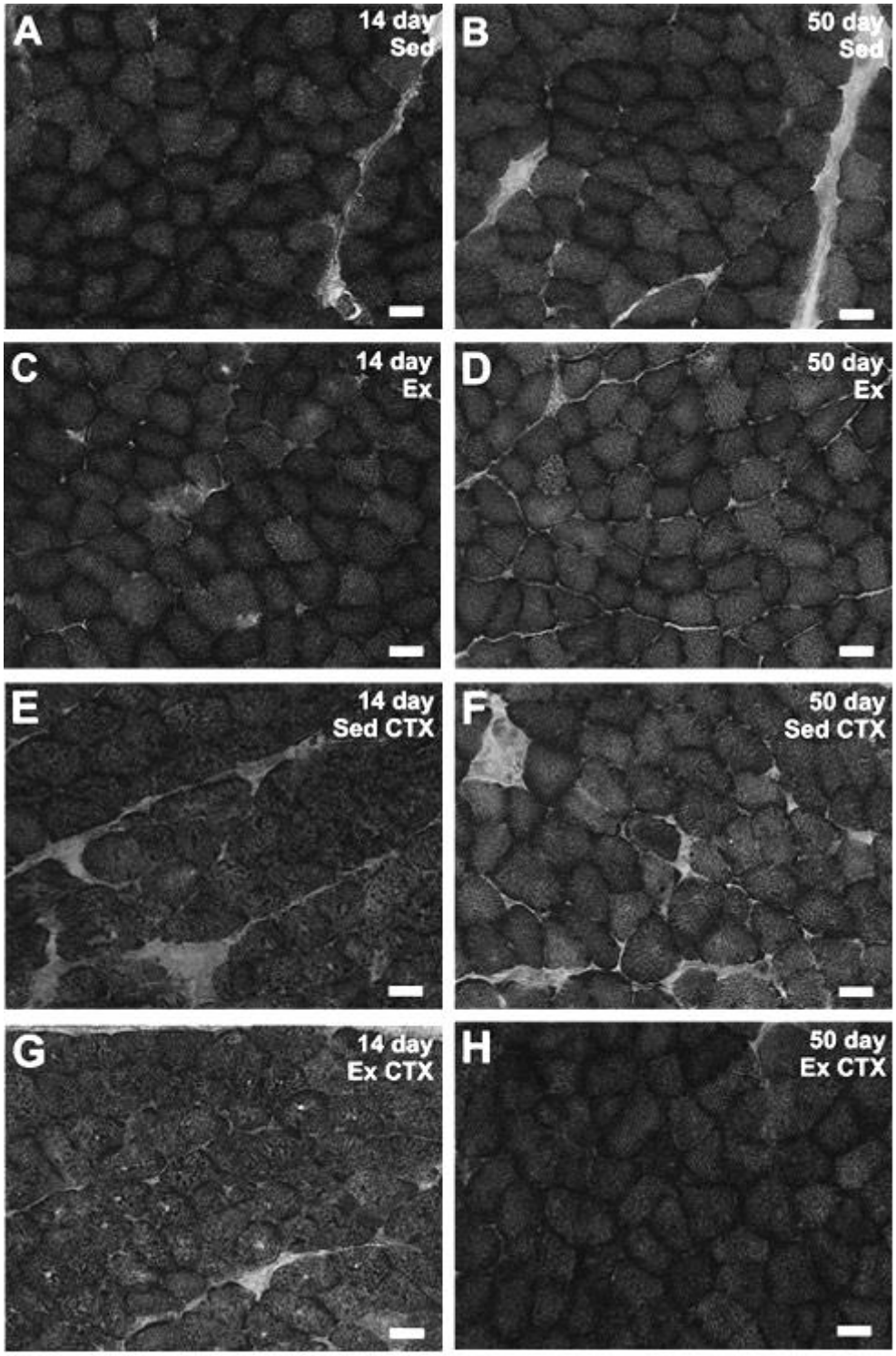

Figure 8. Histochemical SDH stain of mouse soleus muscle cryosections at different timepoints following CTX-induced muscle injury. (A-B) Control (non-injured) soleus of the Sed group at (A) 14 days and (B) 50 days post-CTX injury of the contralateral soleus. (C-D) Control (non-injured) soleus of the Ex-group at (C) 14 days and (D) 50 days post-CTX injury of the contralateral soleus. (E-F) CTX-injured soleus of the Sed CTX group at (E) 14 days and (F) 50 days post-injury. (G-H) CTX-injured

soleus of the Ex CTX group at (G) 14 days and (H) 50 days post-injury. Scale bars represent 50 μm. All images captured at 20x magnification under identical microscope, camera, and brightness settings (e.g., exposure time, gain, binning).

The current study used two weeks of endurance training to induce an effect on muscle regeneration. The training period was followed by a period of de-training to determine if any exercise-induced effects were retained. Interestingly, 36 days after training cessation, SDH activity decreased (main effect of time in all fibre types: $p<0.001$) except for the Ex CTX mice (interaction between time post-CTX injury, exercise, and injury in all fibre types: $p<0.05$; Fig. 7E). SDH activity was significantly higher in the Ex CTX group than the Sed CTX group in the type I ($p=0.002$), IIa ($p=0.003$), IIa/IIx ($p=0.005$), and IIx fibres ($p=0.035$) at 50 days post-injury. While this may be construed as a positive adaptation, it may simply represent a delay in mitochondrial network regeneration. Indeed, the Ex CTX group SDH activity at 50 days was comparable to that of the Sed group at the same time point (Ex CTX= 79.20 ± 8.47 AU, Sed= 75.41 ± 2.68 AU), thus it is difficult to suggest that exercise during regeneration induces a positive, long-term adaptation in mitochondrial function.

The low SDH activity in the Ex CTX mice at 14 days suggests that the mitochondrial regulatory processes were impaired in the exercised regenerating fibres, resulting in dysfunctional mitochondria which will be unable to meet the muscles energy demands. Even within one injured soleus, the regenerating fibres had a visibly weaker SDH stain than those that were uninjured (Fig. 9) These findings suggest that the observed increase in the proportion of type IIa/IIx hybrid fibres in the soleus muscle following injury and exercise training observed by Hian-Cheong (2020) did not coincide with a positive secondary shift in oxidative capacity.

Endurance exercise training during regeneration led to a poorer oxidative capacity in the most oxidative fibre types in mice, the type IIa and IIa/IIx.

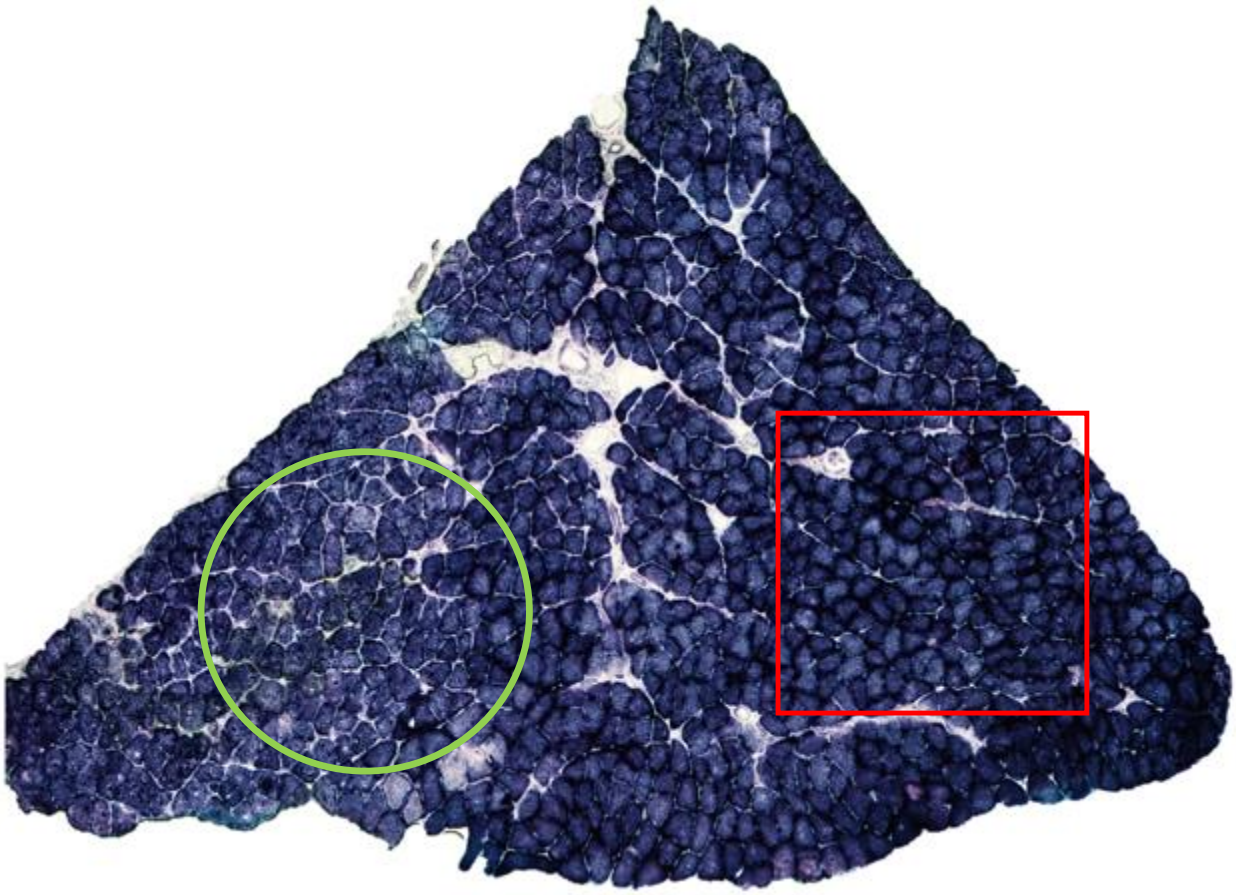

Figure 9. SDH stain of 14-day Ex CTX soleus. Green circle indicating injured (regenerating) region. Red square indicating uninjured region.

There remains the question of why does exercise concurrent with muscle regeneration lead to a slower re-establishment of fibre type and mitochondrial function. In the Ex CTX mice this process may be impaired because of enhanced oxidative stress from the combination of injury and exercise. Although the exercise protocol of this study was not strenuous, the initial increase of ROS at the injury site compounded with the increased ROS from endurance exercise training may have exacerbated oxidative stress in the regenerating fibres, thus delaying their

repair (Høydal et al., 2007; Brickson et al., 2001). Additionally, the energy demands of muscle regeneration, mitochondrial biogenesis and exercise may exceed energy production resulting in delayed mitochondrial biogenesis. An overactive and inefficient AMPK energy sensor may contribute to the lack of energy production as it is activated by decreases to ATP:AMP. Its activation increases ATP generation and mitochondrial autophagy while decreasing protein synthesis (Kjøbsted et al., 2018; Thomson, 2018). The amalgamation of these effects coupled with the ATP demand of endurance exercise may contribute to a delayed regenerative process for the mitochondrial network leading to low SDH activity at 14 days and typical SDH activity at 50 days (Fig. 8G, H).

The muscle regeneration time course has been well-characterized. Damaged fibre breakdown, inflammation, stem cell activity, *de novo* myofibre formation, and neuromuscular junction formation is largely completed within 4-5 days. Establishing mature fibre types normally begins 5-7 days post injury, with the fast MHC expression appearing before slow MHC expression (Ciciliot & Schiaffino, 2010). If innervated by an active slow motor neuron, newly regenerated fast MHC myofibres will switch to slow MHC, establishing its new phenotype (Whalen et al., 1990; Jerkovic et al., 1997). The mitochondrial network should be re-established within 5-10 days of muscle damage (Call et al., 2017; Duguez et al., 2002). After the initial regeneration process, regenerated muscle is in the remodelling phase (Mann et al., 2011). Thus, the current study used a two-week endurance training program to influence the regeneration process prior to muscle remodelling. Typically, two weeks of endurance training would not be expected to induce tremendous changes in mitochondrial activity. Indeed, the current study found similar SDH activity between the Sed and Ex groups at 14 days (Fig. 7E: Sed: 85.77 ± 4.56; Ex: 91.37 ± 5.33 AU, $p = 0.323$). Typically, endurance training studies use 10-12 weeks of

training to induce observable increases in oxidative capacity and/or SDH activity (Chilibeck et al., 1998; Hammeren et al., 1993; Laughlin et al., 1990). Injury was introduced prior to exercise protocol initiation in attempt to accelerate the muscles response to changes in oxidative capacity as previous research has suggested that muscle fibres' phenotype becomes transiently malleable during regeneration (Matsuura et al., 2007). Interestingly, the attempt to induce an oxidative shift via injury and endurance exercise was detrimental to the muscles' oxidative capacity, as the Ex CTX group had the poorest outcome for any group, across all fibre types, especially in the type IIa and type IIa/IIx fibres.

5. LIMITATIONS

A limitation of this study is the exercise training duration. The two-week endurance training protocol may not have been long enough to elicit an alteration in oxidative capacity measured via SDH activity when comparing the sedentary and exercised groups. Injury was introduced to accelerate these adaptations. The current study's injury model injected CTX into the left GPS of each mouse while the right leg remained unharmed. It would have been beneficial to have an additional group that received no CTX and remained sedentary to act as a control group, ensuring the CTX had no unintended effects on the sedentary or exercise group. Additionally, at the 14-day timepoint CTX affected muscle fibres were occasionally limited to the periphery of the soleus which restricted the amount of measurable regenerating regions.

This study solely analyzed alterations in oxidative capacity following injury and endurance training via SDH activity. Previous research has shown that mitochondrial biogenesis and autophagy act as important regulatory events during muscle regeneration (Duguez et al., 2002; Rochard et al., 2000; Call et al., 2017). It may be of interest for future studies to examine

the signalling pathways that govern alterations in mitochondrial biogenesis and their contributions to shifts in oxidative capacity when exercise occurs during regeneration. Specifically, examination of PGC1α in Ex CTX muscle would be relevant due to its participation in both mitochondrial biogenesis and fibre phenotype transitions during exercise (Matsuura et al., 2007). Additionally, future studies may examine the relationship between injury, exercise, aging, and mitochondrial health as differences in oxidative capacity exist between young and old adults regardless of activity level (Grevendonk, 2021). The current study only used male mice to limit the potential influence of fluctuating hormones during the estrous cycle. Recent research has observed sex differences in injury response and fibre type-specific oxidative capacity, therefore examining the effects of injury and exercise on mitochondrial content and oxidative capacity in a sex-specific manner may be of interest (Fournier et al., 2022; Fortino et al., 2022).

5. CONCLUSION

The current study adds to a limited pool of literature that examines fibre type-specific changes to mitochondrial activity and expands upon it by introducing the impacts of injury combined with exercise. This study has found that endurance exercise during muscle regeneration had a negative effect on oxidative capacity in the regenerated type IIa/IIx fibres and IIa fibres. These findings add to previous research where injury and endurance exercise training resulted in more type IIa/IIx hybrids in the regenerated soleus (Hian-Cheong, 2020). Suboptimal mitochondrial performance is associated with numerous pathologies; therefore, the maintenance of mitochondrial content and quality is important for human health. We have shown that immediate return to exercise following injury can negatively impact skeletal muscle mitochondrial content and oxidative capacity in a fibre specific manner.

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