2-29-2016

Effects of Exercise Training and Doxorubicin on Myogenic Regulatory Factors

Colin Joseph Quinn

UNIVERSITYOF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

EFFECTS OF EXERCISE TRAINING AND DOXORUBICIN ON MYOGENIC REGULATORY FACTORS

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

Colin Joseph Quinn

College of Natural and Health Sciences
School of Sport and Exercise Science
Exercise Physiology
December 2015

This Dissertation by: Colin Joseph Quinn Entitled: *Effects of Exercise Training and Doxorubicin on Myogenic Regulatory Factors*

has been approved as meeting the requirement for the Degree of Doctor of Philosophy in the College of Natural and Health Sciences in School of Sport and Exercise Science, Program of Exercise Science

Accepted by the Doctoral Committee

_____

David S. Hydock Ph.D., Research Advisor

_____

Reid Hayward Ph.D., Committee Member

_____

Gary Heise Ph.D., Committee Member

_____

Mark Thomas Ph.D., Faculty Representative

Date of Dissertation Defense: July 22, 2014

Accepted by the Graduate School

_____

Linda L. Black, Ed.D.

Associate Provost and Dean

Graduate School and International Admissions

ABSTRACT

Quinn, Colin Quinn. *Effects of Exercise Training and Doxorubicin on Myogenic Regulatory Factors*. Published Doctoral Dissertation, University of Northern Colorado, 2015.

Doxorubicin (DOX) is a widely used anthracycline antibiotic used to treat a number of hematological and solid tumor cancers. Dosage; however, is limited due to its toxic effects in healthy tissues. Negative consequences include myotoxicity in skeletal muscle, which may limit mobility and activities of daily living. The capacity for skeletal muscular regeneration relies heavily of the activity of myogenic regulatory factor (MRF) proteins. *In vitro* experiments with DOX depress expression of MRFs but *in vivo* treatment may elicit different responses. Endurance exercise has been shown to elevate MRF expression, and may preserve MRFs following *in vivo* DOX-treatment. **Purpose:** To determine the effect of short-term endurance training and acute DOX administration of skeletal muscle force production and fatigue resistance, levels of lipid peroxidation, and expression of MRFs. **Methods:** Ten week old male Sprague-Dawley rats were randomly assigned to one of four groups: sedentary + saline (SED-SAL), SED-DOX, endurance exercise training + saline (EXER-SAL), or EXER-DOX. Animals remained sedentary or performed treadmill training for two weeks. Twenty four hours after the activity period, animals were injected with a bolus 15 mg/kg i.p. injection of DOX or SAL. Twenty four hours after injection, soleus (SOL) and extensor digitorum longus (EDL) skeletal muscles were removed for *ex vivo* function measures. Analyses of lipid peroxidation as malondialdehyde and 4-hydroxyalkenals (MDA + 4-HAE) and

Western blotting for concentration for MRFs (Myf5, MyoD, myogenin, Mrf4) were performed on contralateral muscles.

Results: Endurance exercise significantly elevated Myf5 and Mrf4 in the SOL ($p<0.05$). No significant differences existed in MRF expression levels in the EDL. No significant muscle force production or fatigue resistance differences were identified due to drug or activity treatment. MDA + 4-HAE was higher in the SOL of SAL animals ($p<0.05$) and EDL of EXER animals ($p<0.05$). **Conclusion:** Short-term endurance exercise effectively elevated Myf5 and Mrf4 in slow, oxidative muscle after acute DOX treatment. Endurance exercise prior to chemotherapy may augment skeletal muscles' regenerative capacity following treatment, when loss of muscle mass is common.

TABLE OF CONTENTS

CHAPTER

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

CHAPTER I

INTRODUCTION

Background

Muscle weakness and subsequent deterioration in activities of daily living are common side effects of chemotherapy treatments prescribed to cancer patients (Bonifati et al., 2000; Burckart, Beca, Urban, & Sheffield-Moore, 2010; Knobel et al., 2001). The commonly used anthracycline antibiotic, doxorubicin (DOX; trade name: Adriamycin®) has been shown to cause severe to fatal consequences associated with its cardiotoxic nature. Free radicals formed by iron-catalyzed reactions are implicated in nuclear and mitochondrial damage inducing cell death (Bagchi, Bagchi, Hassoun, Kelly, & Stohs, 1995; DeAtley et al., 1999; Rapozzi et al., 1998; Stathopoulos et al., 1997). Much of the existing literature surrounding DOX-induced injury focuses on effects seen in the heart. More recently, research has elucidated serious skeletal muscle harm, decreasing muscle size and function in response to DOX exposure (Doroshow, Tallent, & Schechter, 1985; Gilliam et al., 2009; Gilliam et al., 2013; Gilliam, Moylan, Callahan, Sumandea, & Reid, 2011). Previous research has demonstrated the beneficial influence of endurance exercise preconditioning in mitigating the negative cardio- and myotoxic consequences of DOX treatment (Ascensão, Oliveira, & Magalhães, 2012; Chicco, Schneider, & Hayward, 2006; Hayward, Lien, Jensen, Hydock, & Schneider, 2012; Hydock, Lien, Jensen, Schneider, & Hayward, 2011b).

Skeletal muscle comprises a large part of the human body, responsible for posture and locomotion. It is unique in its plasticity to alter its form following various stimuli. In adult muscle, myogenic regulatory factors (MRFs) guide satellite cells to restore muscle integrity in response to damage and stress, such as exercise. Activated satellite cells, expressing primary MRFs, form myoblasts, and differentiate into myotubes in response to secondary MRFs and reconstitute muscle fibers. The ability of skeletal muscle to repair itself and retain structure relies heavily on functional MRF proteins.

In vitro DOX exposure has been shown to decrease the ability of myoblasts to differentiate into myotubes (Kurabayashi, Jeyaseelan, & Kedes, 1993). Additionally, MRF mRNA expression is compromised under the same conditions with an up-regulation of the MRF inhibitor, Id (Kurabayashi, Jeyaseelan, & Kedes, 1994). DOX has been shown to induce oxidative stress, leading to cellular damage and single-stranded DNA breaks. Beyond oxidative damage, genotoxic stress attributed to DOX leads to double-stranded DNA breaks. The primary MRF, MyoD, is fundamentally involved in myoblast DNA repair (Kobayashi, Antoccia, Tauchi, Matsuura, & Komatsu, 2004). Its presence, along with the other MRFs, is critical for skeletal muscle regeneration following chemotherapy treatment including DOX. Although MRF mRNA has been broadly investigated, functional protein expression has been less examined. Functional MRF protein may be enhanced with endurance exercise prior to drug treatments.

In a pilot study (see Appendix B), rat skeletal muscle was examined three days following DOX or saline injections. Sedentary animals treated with DOX versus saline injections displayed differential expressions of MRFs in soleus (SOL) and extensor digitorum longus (EDL) muscles. Myf5 and MyoD were lower in SOL of animals

receiving DOX, while myogenin and Mrf4 were significantly greater. Myf5 and MyoD increased in EDL and Mrf4 decreased following DOX injections. Prior endurance exercise training may influence the expression of MRFs in skeletal muscle. Following endurance training, SOL of rats demonstrated elevated levels of myogenin (Siu, Donley, Bryner, & Alway, 2004). In combination with elevated antioxidant enzymes due to short-term aerobic exercise, increased MRF protein may effectively mitigate the skeletal muscle dysfunction attributed to DOX treatment and enhance subsequent repairs.

Statement of Purpose

The purpose of this study was to investigate the effects of *in vivo* DOX administration on skeletal muscle force production and fatigue resistance, oxidative damage, and expression of MRFs (MyoD, Myf5, myogenin, & Mrf4). A secondary purpose of the study was to examine if prior short-term exercise provides protection against DOX-induced muscle dysfunction, lipid peroxidation and MRF alterations in skeletal muscle. Whether MRF protein expression affects muscle function or is, itself, affected by oxidative stress may be elucidated with this research. The study addressed myogenic mechanisms associated with adult muscle regenerative capacity occurring with DOX treatment. An additional goal of the study was to identify whether short-term aerobic exercise intervention can be used to offset muscular dysfunction afflicting cancer patients.

Research Hypotheses

Specific Aim 1

The purpose of the first specific aim was to identify the effects of DOX treatment and 2-week endurance training on *ex vivo* skeletal muscle force production and fatigue resistance.

H1 DOX treatment will impair skeletal muscle twitch force production and fatigue resistance when analyzed 1 day following DOX injection.

H2 Short-term endurance exercise will minimize DOX-induced myotoxic function.

Specific Aim 2

The second specific aim was to determine oxidative stress in response to DOX and exercise in skeletal muscles.

H3 DOX treatment will increase lipid peroxidation levels 1 day following DOX injection.

H4 Short-term endurance exercise will minimize DOX-induced lipid peroxidation in both SOL and EDL.

Specific Aim 3

The third specific aim was to identify the effect of short-term exercise and acute, *in vivo* DOX treatment on MRF expression in hindlimb muscles.

H5 Primary MRF protein (Myf5 and MyoD) concentrations will significantly decrease in slow, oxidative muscles (SOL) and increase in fast, glycolytic muscles (EDL) following *in vivo* DOX treatment compared with control animals.

H6 Secondary MRF protein (myogenin and Mrf4) concentrations will significantly increase in SOL and decrease in EDL following *in vivo* DOX treatment compared with control animals.

H7 Prior endurance exercise will attenuate MRF alterations associated with DOX treatment.

Need for Study

Skeletal muscle weakness following chemotherapy is well documented. Decreases in activities of daily living and susceptibility to falls can have dire consequences. Recent literature has shown time to chemotherapy can have adverse outcomes, especially with higher graded cancers (de Melo Gagliato et al., 2014). Although research has demonstrated chronic endurance exercise to provide a protective effect, time does not often allow for an extended exercise-training period. DOX prescription in chemotherapy is quite common, and patients often experience later cardiotoxicity, but patients also exhibit skeletal muscle degeneration and dysfunction. The benefit of exercise preconditioning has proven useful in protecting cardiac and skeletal muscles from ills associated with DOX treatment, but time to chemotherapy may not afford such a time until treatment. Designing a useful, short-term aerobic exercise intervention to maintain skeletal muscle form and function may improve the adaptive capacity of muscle following DOX treatment. Myogenic regulatory factors play an integral role in the regeneration of adult muscle tissue, and methods to maintain their expression may afford greater quality of life following chemotherapy. Short-term exercise preconditioning may offer skeletal muscles the capability to combat the oxidative and genotoxic stress associated with doxorubicin treatment.

Table 1.1

Abbreviations

4+HAE = 4-Hydroxyalkenals	ABL = Abelson murine Leukemia
ANOVA = Analysis of Variance	Bax = Bcl-2-associated X protein
BCA = Bicinchronic Acid	bHLH = Basic Helix-Loop-Helix
C2C12 = Mouse myoblast cell line	Ca^{2+} = Calcium
Caspase = Cysteine-aspartic protease	CHF = Congestive Heart Failure
CO = Carbon monoxide	DDR = DNA Damage Response
DNA = Deoxyribonucleic Acid	DOX = Doxorubicin
DSB = Double-Strand Break	ECL = Enhanced Chemiluminescence
EDL = Extensor Digitorum Longus	EXER = Exercise
GPX = Glutathione Peroxidase	H_2O_2 = Hydrogen Peroxide
HRP = Horseradish Peroxidase	Hz = Hertz
i.p. = Intraperitoneal	Id = Inhibitor of DNA binding
$K^{+ =}$ = Potassium	LPO = Lipid Peroxidation
mA = Milliampere	MCK = Muscle Creatine Kinase
MDA = Malondialdehyde	MEF2 = Myocyte Enhancer Factor 2
MPC = Myogenic Progenitor Cell	MRF = Myogenic Regulatory Factor
mRNA = Messenger RNA	MyHC = Myosin Heavy Chain
Nbs1 = Nijmegen Breakage Syndrome 1 gene	$O_2^{\bullet -}$ = Superoxide Anion
$OH^{\bullet}$ = Hydroxyl radical	Pax = Paired-homeobox
PUFA = Polyunsaturated Fatty Acid	PVDF = Polyvinylidene Fluoride
RIPA = Radioimmunoprecipitation Assay	RNA = Ribonucleic Acid
ROO^{-} = Peroxyl	ROS = Reactive Oxygen Species
SAL = Saline	SC = Satellite Cell
SDS-PAGE = Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis	SED = Sedentary
SHH = Sonic Hedghog	SOD = Superoxide Dismutase
SOL = Soleus	SR = Sarcoendoplasmic Reticulum
SSB = Single-Strand Break	TBST = Tris-Buffered Saline with Tween20
TM = Treadmill	TNF = Tumor Necrosis Factor
TOP2 = Topoisomerase II	V = Volt

Definition of Terms

Anthracycline – Class of antibiotic used in chemotherapy derived from Streptomyces bacterium

Antineoplastic – Preventing the growth and spread of tumors or malignant cells

Apoptosis – Process of programmed cell death, marked by morphological changes and DNA fragmentation

Caspase – Cysteine protease involved in direction of apoptosis, necrosis, and inflammation

Double-strand break – Condition where both strands of DNA double helix have been individually cleaved without separation of the two strands

Doxorubicin – Bacterial antibiotic used in the treatment of various cancers

Embryogenesis – Formation and development of an embryo

Genotoxic stress – Damage to the genome of an organism as result of a genotoxin

Genotoxin – Substance capable of causing damage to cellular DNA and causing mutations or cancer

Myogenesis – Formation and growth of muscle tissue

Oxidative stress – Damage caused to cells or tissue as result of reactive oxygen species

Redox cycling – Reactions involving the transfer of electrons, resulting in change between reduced and oxidized states

Single-strand break – Cleavage of only one of the two strands of DNA, while both strands remained attached to one another

Zymogen – Inactive enzyme precursor

CHAPTER II

REVIEW OF LITERATURE

Doxorubicin

Doxorubicin (DOX; trade name: Adriamycin®) is a widely used antineoplastic for treating both solid and hematological cancers. Clinically, DOX is administered intravenously (i.v.) and in some cases directly into the abdomen via intraperitoneal (i.p.) injections (Chabner, Ryan, Paz-Ares, & Garcia-Carbonero, 2001; Chu & DeVita, 2006; Sugarbaker, 2009; Van der Speeten, Stuart, Mahteme, & Sugarbaker, 2009). Unfortunately, DOX use is limited due to undesirable effects on cardiac muscle, including contractile dysfunction, dilated cardiomyopathy, and congestive heart failure (CHF) (Singal, Li, Kumar, Danelisen, & Iliskovic, 2000; Singal & Iliskovic, 1998). Additionally, skeletal muscle dysfunction is observed following DOX administration. DOX-induced skeletal myopathies can lead to severe respiratory and locomotor impairments (Kavazis, Smuder, & Powers, 2014).

The proposed mechanisms behind the antineoplastic action of DOX suggest that DOX inhibits DNA synthesis, forms free radicals, promotes lipid peroxidation, binds and alkylates DNA, interferes with DNA separation activity, directly affects cell membranes, initiates DNA damage via topoisomerase II, and signals apoptosis (Gewirtz, 1999; Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). Anthracyclines, like DOX, are prone to generate reactive oxygen species (ROS) when mitochondrial enzymes interact

with a quinone ring C of DOX (Figure 2.1), releasing electrons that are captured by oxidizing agents (i.e., oxygen). Molecular oxygen is then reduced to ROS like superoxide, hydroxyl and peroxide radicals. Elevated levels of ROS lead to lipid peroxidation, DNA damage and cell apoptosis, arresting malignant cancer growth. These effects, however, are not limited to cancerous cells, and healthy cells are also affected. Because of this unwanted side effect, dosages of DOX are limited to minimize side effects.

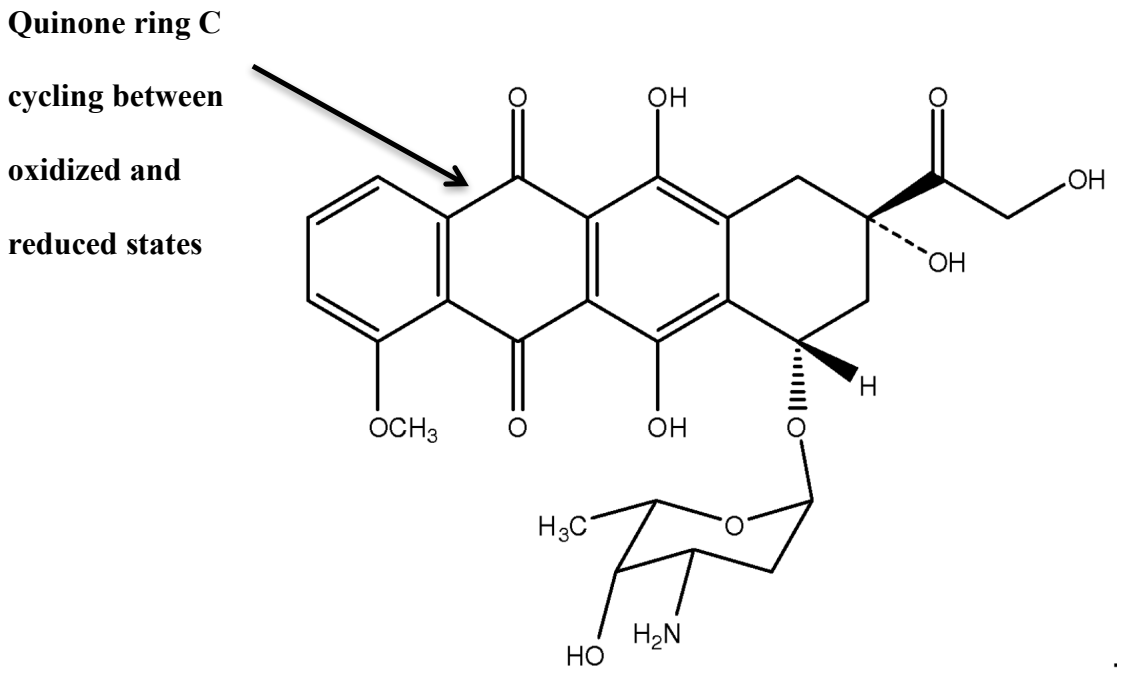

Figure 2.1. Structure of DOX (Minotti et al., 2004)

Reactive Oxygen Species

Oxidative stress is a condition in which the cellular balance of pro-oxidants outweighs antioxidants. Oxidative stress primarily occurs when oxygen free radicals accumulate within the body. Oxygen free radicals are small, diffusible molecules with an

unpaired electron, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), peroxyl (ROO^-), or hydroxyl radical ($OH^\bullet$). Accumulated oxidative damage has been shown to induce mitochondrial damage and apoptosis, while furthering the formation of ROS (Valko et al., 2007).

Any protein or enzymatic system, which transfers electrons, can form ROS by donating or receiving electrons from oxygen or oxygen-derived compounds. Therefore, ROS byproducts can be found in mitochondria, peroxisomes, cytochrome p450 reactions and phagocytic immune responses. Flavoenzymes use NADPH as an electron source in redox cycling. An electron is transferred from NADPH to the quinone of anthracyclines, reducing it to a semiquinone state. The semiquinone rapidly returns to its parent state once the electron is donated to oxygen, forming superoxide ($O_2^{\bullet-}$) (Gutierrez, 2000). Following DOX administration, ROS are produced largely in the mitochondria, with redox cycling at complex I of the electron transport chain (Minotti et al., 2004). At this stage, DOX is converted to a semiquinone reactive, forming $O_2^{\bullet-}$ and subsequent H_2O_2 and $OH^\bullet$ (Davies & Doroshow, 1986; Doroshow & Davies, 1986).

In the normal physiological environment, oxygen free radicals are produced as part of oxidative metabolism. In addition to inducing apoptosis, ROS can signal gene expression as second messengers. ROS production is a major regulator of signaling pathways promoting skeletal muscle adaptations following exercise (Franco, Odom, & Rando, 1999; Li, Chen, Li, & Reid, 2003). The levels of ROS, however, must be maintained at homeostatic levels to allow cellular processes of muscle growth and differentiation. Although exercise leads to a spike in oxygen free radical production, antioxidant enzymes are increased to better handle radicals and shift levels toward a

healthy balance. The naturally occurring antioxidant enzymes in skeletal muscle include catalase, glutathione peroxidase (GPX), and superoxide dismutase (SOD). Under normal conditions, ROS generation is significantly higher in type IIB muscle fibers than type I or type IIA, whereas ROS clearance rates are highest in type I fiber due to higher levels of antioxidant enzymes (Anderson & Neufer, 2006). During conditions of inflammation and disease, ROS concentrations are elevated beyond the capacity of antioxidant handling.

ROS also affect the regulation of K^+ channels, plasma membrane Ca^{2+} channels and intracellular Ca^{2+} channels in muscle tissues (Hool, Di Maria, Viola, & Arthur, 2005; Tang, Santarelli, Heinemann, & Hoshi, 2004). Maintenance of redox potential in myocytes is important because hypoxic conditions will inactivate K^+ channels, decreasing carbon monoxide (CO) production and muscle metabolism while increasing ROS generated. This condition fails to maintain redox homeostasis and results in cell injury or dysfunction (Hoshi & Lahiri, 2004).

Uncontrolled oxidative stress acts as a feed-forward process, whereby the increased concentration of ROS enhances intracellular and extracellular production of ROS. Subsequent deleterious effects include lipid perodixation, damage to cell membranes and organelles, disrupted signaling pathways, apoptosis, and muscle atrophy. Lipid peroxidation (LPO) is a chain reaction initiated and furthered by oxygen free radicals. ROS target double bonds of polyunsaturated fatty acids (PUFAs), and compromise the integrity of mitochondrial membranes, which contain high levels of PUFAs (Chance, Sies, & Boveris, 1979).

When these mitochondrial membranes become perforated due to the pro-oxidant state, cytochrome c is released into the cytosol inducing caspase 9 signals for intrinsic

cell apoptosis. Once inside the cytosol, cytochrome c binds to apoptotic protease activating factor 1, allowing for the additional binding of a pro-caspase 9 zymogen in the presence of adenosine triphosphate (ATP). This newly formed complex autoactivates caspase 9, and signals caspase 3 downstream, inducing programmed cell death. Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) may activate the p53 gene inducing apoptotic events as well. p53 directly activates the Bax gene (apoptotic factor), which binds to a mitochondrial membrane-bound receptor and opens channels leading to a release of cytochrome c and pro-caspase 9 (Chandra, Samali, & Orrenius, 2000; Feng Gao et al., 2001). This mitochondrial (intrinsic) pathway differs from the death receptor pathway, whereby signals, such as TNFα, bind to a plasma membrane-bound Fas ligand receptor, and stimulate apoptosis via caspase 8 (Fulda & Debatin, 2006). Additionally, activated p53 translocates to the nucleus where it induces gene expression (p21) preventing cell division (Cui, Schroering, & Ding, 2002).

Deoxyribonucleic Acid Damage

Beyond membrane damage, uncontrolled oxidative stress may augment or individually disrupt deoxyribonucleic acid (DNA) transcription and synthesis. Hydroxyl radicals ($OH^{\bullet}$) have been shown to cause DNA base modification or fragmentation (Van Remmen, Hamilton, & Richardson, 2003). Disruption of the genome and replication processes following radiation and chemotherapy is referred to as "genotoxic stress" (Simonatto et al., 2011). The main forms of DNA damage likely to occur following DOX administration are ROS-induced single-strand break (SSB), topoisomerase II-mediated double-strand break (DSB), and apoptotic DNA fragmentation (Swift, Rephaeli, Nudelman, Phillips, & Cutts, 2006). DOX treatment causes early event H_2O_2

accumulation, which may be responsible for oxidative DNA lesions in cardiomyocytes (L'Ecuyer et al., 2006). These oxidative lesions are readily repaired upon removal of oxidants by free radical scavengers, such as N-acetyl cysteine (L'Ecuyer et al., 2006). Non-oxidative, DOX-induced DNA damage, however, is less prone to rapid repair. Specifically, anthracycline-induced DNA lesions include oxidized pyrimidines and 8-hydroxyguanine (L'Ecuyer et al., 2006).

When DNA templates are damaged (i.e., lesions), cells stall at checkpoints before replication and mitotic chromosome separation at the transition from G_1 to S phase and G_2 to M phase (fork stalls). These delays facilitate the maintenance of proper DNA sequences and prevention of accumulated DNA alterations (Nelson & Kastan, 1994). At the G_1 checkpoint, the tumor suppressor protein, p53, acts as a major regulator. Following DNA damage, p53 levels rapidly increase and signal transduction pathways lead to G_1 cycle arrest or apoptosis (Kastan, Onyekwere, Sidransky, Vogelstein, & Craig, 1991). In myoblasts, p53 transactivates p21 to arrest the cell cycle following DOX treatment. Terminally differentiated myotubes, however, do not exhibit p53 activation of p21 or Bax genes, yet cell death occurs when exposed to DSB-inducing agents, like DOX (Fortini et al., 2012).

Intercalative antibiotics, such as DOX, generate protein-linked DNA strand breaks via topoisomerase II (TOP2) inhibition (Tewey, Rowe, Yang, Halligan, & Liu, 1984). Topoisomerases are enzymes that regulate the intertwining of DNA double-helical structure. Mammalian TOP2 catalyzes topological isomerization reactions, via binding DNA and passing the DNA strand to correct base pair structures and over-winding. The TOP2-DNA union is referred to as a “cleavable complex.” DOX stabilizes these

topoisomerase-DNA complexes, stalling replication forks. The stabilization of the "cleavable" TOP2-DNA complex pauses strand passing activity, and prevents DNA replication and RNA synthesis (Tewey et al., 1984). Beyond stabilizing the complex, DOX intercalates elsewhere on DNA, binding tightly and further damaging strand integrity. Furthermore, inability to cleave the stabilized complex leaves a lesion, which results in a DNA DSB (Swift et al., 2006). In response to DNA damage, an apoptotic reaction is signaled.

DNA damage response (DDR) is a complex network involving repair factors and cell cycle regulators when genotoxic stress is induced (Ciccia & Elledge, 2010). Checkpoints for DNA damage repair are at transitions from G_1/S and G_2/M phases (Simonatto et al., 2011). If repair is not successfully completed, cells are destined to apoptotic fates. Oxidative stress in terminally differentiated muscle cells may decrease base excision repair (BER) capacity and lead to accumulation of SSBs, but cell death is not inevitable in post-mitotic cells. DOX-induced muscle cell death is mainly attributed to the activation of p53 by topoisomerase II DSB (Fortini et al., 2012). Similarly, Müller and colleagues (1997) report apoptosis in skeletal muscle to be dependent on RNA synthesis disruption rather than oxidative damage, which requires higher concentrations to signal apoptosis. When DNA chromatin is damaged, histone H2AX is phosphorylated and forms foci at the injured site (Kobayashi et al., 2002).

Doxorubucin and Skeletal Muscle

The toxic effects of DOX on cardiac muscle are well documented, but similar parallels in characteristics of DOX-induced changes have been shown following single i.p. DOX injections with losses in skeletal muscle mass and myofibrillar disorganization

(Doroshow, Tallent, & Schechter, 1985). Both Type 1 and Type 2 muscle fibers decrease in size following single limb DOX perfusion (Bonifati et al., 2000). Additionally, DOX may upset excitation-contraction (EC) coupling mechanisms by altering SR Ca^{2+} transport, thereby reducing muscle force production (Hidalgo, Bull, Behrens, & Donoso, 2004; van Norren et al., 2009). Studies investigating cardiac muscle have shown no changes in Ca^{2+} sensitivity, but found inhibition of SR uptake and release following DOX treatments (Chugun et al., 2000).

Our lab demonstrated that skeletal muscle, similar to cardiac tissue, exhibited severe functional declines in a time-dependent fashion following DOX injections (Hayward et al., 2013). Additionally, proteolysis of skeletal muscle occurs following DOX injections, resulting in degradation of myofibrillar actin (Smuder, Kavazis, Min, & Powers, 2011b). Subsequently, this proteolysis may be linked to decreased muscle mass, as muscle cross-sectional area is reduced following DOX administration (Mcloon, Falkenberg, Dykstra, & Iaizzo, 1998). Functionally, patients who were treated with DOX displayed reduced handgrip strength and quicker rates of fatigue (Stone et al., 1999). Although i.p. DOX treatment does not affect limb muscles as severely as the heart or diaphragm, cellular disruption, catabolism, and functional declines are evident (Doroshow et al., 1985; Gilliam et al., 2009; Gilliam et al., 2012). Furthermore, DOX treatment down-regulates contractile gene expression to include myosin-binding protein H, myosin light chain 4, and troponin T type 1 (Simonatto et al., 2011). Clinical DOX treatment clearly initiates skeletal myotoxic consequences.

Skeletal Muscle Growth

Skeletal muscle is a dynamic, plastic tissue adept to hypertrophy in response to growth factors, nutrition, and exercise (Evans, 2004). The growth and development of skeletal muscle is mediated by a process whereby myoblasts produce myogenic regulatory factors (MRFs), which allow further development and differentiation of a large number of different cell types into muscle (Lowe & Alway, 1999; Megeney & Rudnicki, 1995). The MRFs are a family of basic helix-loop-helix (bHLH) muscle specific regulatory proteins expressed exclusively by myonuclei and activated by satellite cells. MRFs contain a basic region that facilitates DNA binding and HLH domain mediating dimerization (Davis, Cheng, Lassar, & Weintraub, 1990). The HLH motif in MRFs consists of α-helices, separated by a variable loop region. The consensus DNA sequence 5'-CANNTG-3', referred to as an "E-box," serves as the DNA target site for the basic region (Murre et al., 1989). Activation of gene expression by MRFs requires heterodimerization with another bHLH protein, which is typically from the E-protein family. The bHLH E-proteins, E12 and E47, are 2 splice variants of the E2A gene that are ubiquitously expressed and most frequently dimerize with MRFs (Lassar et al., 1991).

Once MRF-E protein heterodimers are formed, the DNA-binding domain binds to a target E-box (CANNTG) site. The E-box sequence of DNA is found in the regulatory region of many skeletal muscle-specific genes (Siu, Donley, Bryner, & Alway, 2004). Downstream targets are then activated, leading to the expression of muscle structural genes (contractile proteins) and generation of differentiated muscle phenotypes. Additionally, the MRFs interact with MEF2 family of MADS-box transcription factors, including MEF2A, -B, -C and –D. Not all skeletal muscle genes have E-boxes, and may

require other factors to activate transcription (Hauschka, 1994). MEF2 factors bind to MRF basic domains increasing conversion of non-muscle cells with MRFs (Molkentin, Black, Martin, & Olson, 1995). MRFs autoregulate their own expression and induce MEF2 expression. MEF2 factors bind to promoters of myogenic bHLH genes, reinforcing their maintenance and transcription in a positive feedback fashion (Olson, Perry, & Schulz, 1995).

MRFs include the transcription factors: MyoD, Myf5, myogenin and Mrf4. These transcription factors act by increasing satellite cell proliferation and differentiation into myoblasts. Functions of MRFs overlap, but are distinct in function with one rescuing myoblast development in the absence of individual factors. MyoD and Myf5 are expressed in proliferating myoblasts, and are referred to as primary MRFs. Myogenin and Mrf4, or secondary MRFs, are present in myocytes in the process of terminal differentiation. MyoD and Myf5 are expressed initially in proliferating myoblasts and differentiating muscle, promoting entry into the cell cycle (Sabourin & Rudnicki, 2000). Myogenin and Mrf4 are expressed later following myocyte fusion, promoting differentiation and cell cycle exit (Hinterberger, Sassoon, Rhodes, & Konieczny, 1991).

Upstream of MRFs, the paired-box (Pax) family of transcription factors signal the activation of satellite cells for growth and repair of skeletal muscles. Specifically, Pax3 and Pax7 are expressed uniquely in satellite cells. Upon injury, Pax7-expressing satellite cells migrate to the damaged tissue. The Pax transcription factor levels are reduced with concurrent increase of Myf5 and MyoD, leading to differentiation of myoblasts (Braun, Rudnicki, Arnold, & Jaenisch, 1992; Rudnicki et al., 1993). Cells expressing Myf5 and MyoD expand and proliferate myoblasts. After cue for differentiation, myoblasts exit the

cell cycle as myotubes and express myogenin and p21 (Halevy et al., 1995). The terminally differentiated, multinucleated myotubes are distinguished by expression of myosin heavy chain (MyHC) and troponin T.

In the event that p53 signals nuclear apoptosis in skeletal muscle cells, the whole myofiber is not necessarily degraded. Due to the multinucleated nature of skeletal muscle fibers, individual myonuclear apoptosis can occur without complete cell death (Primeau, Adhihetty, & Hood, 2002). The nuclei selected for apoptosis are degraded without affecting the survival of other nuclei in the shared cytoplasm or causing cell destruction (Primeau et al., 2002).

Satellite Cells

The regenerative properties of skeletal muscle are due to progenitor cells known as satellite cells (SCs). In adult muscle, SCs are small mononucleated skeletal muscle precursor cells between the basal lamina of the muscle and sarcolemma of myofibers. In adult tissue, approximately 5% of myonuclei in muscle fibers are within SCs (Bischoff, 1994; Zammit & Beauchamp, 2001). These unstressed cells are quiescent and arrested at an early stage of the myogenic program. Following injury or in response to increased functional demand, SCs become activated, rapidly divide, and exit the cell cycle. Next, SCs migrate to the site of injury, proliferate, and differentiate into myoblasts. Cytoplasmic volume increases and organelles develop, and the SC nuclei become myonuclei. The myoblasts fuse to one another, forming multinucleated myotubes, or fuse in place of damaged fibers, repairing damaged myofibers (Moss & Leblond, 1971). Some of the proliferating SCs do not differentiate but rather remain quiescent in the G_0/G_1 phase of the cell cycle. Adult myonuclei are unable to divide, so repair and growth of

muscle depends on the availability of SCs to provide new myonuclei in adult muscle fiber. Due to the insulated position of SCs, they are shielded from environmental exposure such as genotoxic stress experienced with DOX (Pallafacchina et al., 2010).

Frequency of SCs differs among muscle fiber types, where red, oxidative fibers have more than white, glycolytic fibers. Additionally, myonuclear density is higher in the red fibers, likely due to higher metabolic activity. Although MRFs can induce other cell types (fibroblasts, etc.) to a myogenic fate, SCs provide the largest pool of new myogenic precursors in hypertrophying muscle fibers (Moss & Leblond, 1971; Schiaffino, Bormioli, & Aloisi, 1976).

Pax7 is a transcription protein expressed in cells that lie beneath the basal lamina, and Pax7-null animals have no SCs (Seale et al., 2000). A number of SCs retain their Pax7 expression while down-regulating MyoD, reversibly exit the cell cycle, and reposition to the basal lamina until needed (Zammit et al., 2004). Continued Pax7 expression inhibits the pathway to myogenic fate (Wang & Conboy, 2010). After activation and prior to differentiation, SCs express either Myf5 or MyoD (Cornelison & Wold, 1997).

Somite Myogenesis

Vertebrate skeletal muscle is derived from somites formed during embryogenesis. Somites are epithelial structures segmented from the paraxial mesoderm. The dorsal portion of the somite is referred to as the dermomytome, which is comprised of dermal and muscle progenitor cells (MPCs), whereas the ventral side will form the vertebral column and ribs. The edges of the dermomyotome transitions from epithelial to mesenchyme tissue, giving rise to the myotome, where the first differentiated myofibers

exist. The dorsomedial (epaxial) region of the dermomyotome and myotome will become the axial muscles of the back, while the ventrolateral (hypaxial) somite will generate other trunk and limb muscles (Buckingham, 2001). At this stage, MPCs do not express any MRFs or other skeletal muscle markers. Myogenesis in the myotome is induced by both the notochord and neural plate cooperatively through members of the Wnt family of growth factors to include Sonic hedgehog (Shh), Wnt-1, -3, and -4 proteins. Somatic cell precursors, originating from the dermomyotome, migrate to limbs to commit to myogenic fates (Brand-Saberi & Christ, 1999).

Gene ablation models have determined much of what is known about myogenesis, whereby genes are deleted to determine the function served. MPCs are distinguished by the presence of Pax3 and Pax7. When Pax3 fails to be expressed, muscle development is impaired, with a complete loss of the hypaxial somite and loss of limb and some trunk muscles (Dietrich, Schubert, Gruss, & Lumsden, 1999; Relaix, Rocancourt, Mansouri, & Buckingham, 2004). In the absence of Pax3, epaxial muscles are less affected, but death occurs mid-gestation (Chi & Epstein, 2002). Later in development, Pax7 becomes more important, as MPCs cannot begin myogenic development without it. Lack of both Pax3 and Pax7 in MPCs will not allow entrance to myogenic lineage. The four bHLH transcription factors, MyoD, Myf5, myogenin, and Mrf4, follow the Pax genes in the myogenic development of the myotome.

Myogenic identity of cells is signaled by the determinant transcription factors, Myf5 and MyoD. Individual disruption of Myf5 or MyoD in mice delays formation of hypaxial and epaxial muscles, but no gross defects are evident in differentiation due to functional redundancy of primary MRFs (Braun et al., 1992; Rudnicki, Braun, Hinuma,

& Jaenisch, 1992). With losses in both Myf5 and MyoD, mice show reduced muscle mass due to defective myoblast formation (Rudnicki et al., 1993). Additionally, severe defects occur when differentiating secondary factors fail to be expressed. When myogenin is absent in mice, myofiber formation is compromised causing animals to die perinatally (Hasty et al., 1993; Nabeshima et al., 1993; Venuti, Morris, Vivian, Olson, & Klein, 1995). Failure to express the Mrf4 gene leads to subtle deficiencies in myogenesis such as reduced muscle specific genes, but no major defects in muscle development occurs, likely due to overlapping functions of other factors (Olson, Arnold, Rigby, & Wold, 1996).

Myogenic Regulatory Factors

Similar to the development of muscle during embryogenesis, muscle regeneration in response to injury is highly dependent on the proliferative response of satellite cells expressing myogenic regulatory factors (MRFs) to reconstruct functional myofibers. In adult muscle, SCs follow similar genetic programming exhibited in embryonic myogenesis, acting comparably to MPCs (Rudnicki, Le Grand, McKinnell, & Kuang, 2008). When satellite cells become committed to myogenic fate, they express both Myf5 and MyoD. In these active, proliferating myoblasts, early differentiation begins with the expression of myogenin, followed by Mrf4. Cells are now referred to as myocytes, which begin late differentiation, and form myotubes and ultimately fuse into myofibers (Figure 2.2) (Bentzinger, Wang, & Rudnicki, 2012). During late differentiation, myocytes also express genes for muscle creatine kinase (MCK) and myosin heavy chain (MyHC), responsible for contractile structure (Karalaki, Fili, Philippou, & Koutsilieris, 2009).

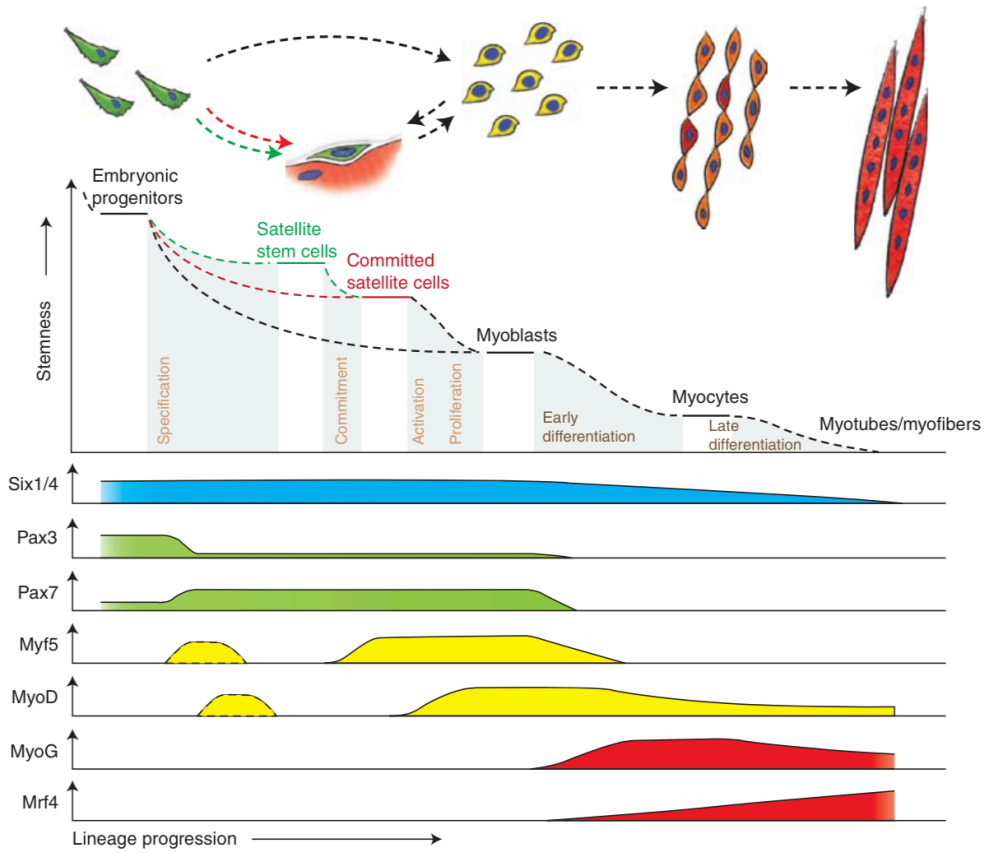

Figure 2.2. Hierarchy of transcription factors regulating progression through the myogenic lineage (Bentzinger et al., 2012)

Myogenic Factor 5

Myogenic factor 5 (Myf5) is the earliest expressed MRF in skeletal myogenesis (Zweigerdt, Braun, & Arnold, 1997). Myf5, along with MyoD, is required for determination to myogenic lineage. Myf5 interacts with Pax3 upstream of MyoD and may be responsible for specifying muscle cell types (Tajbakhsh, Rocancourt, Cossu, & Buckingham, 1997). Pax7 indirectly up-regulates Myf5 expression, inducing myoblast proliferation (McKinnell et al., 2008). Decreased Myf5 expression early in myogenesis inhibits differentiation. Defects in Myf5 expression have been shown to decrease development of rib muscles and lead to perinatal or neonatal death, as evidenced by

Myf5$^{-/-}$ knockout mice (Braun et al., 1992). Myf5-null myoblasts have been shown to proliferate poorly and differentiate quickly (Montarras, Lindon, Pinset, & Domeyne, 2000). A predominant Myf5 expression versus MyoD in adult muscle has been shown to direct greater myoblast proliferation and delayed differentiation, which may be responsible for satellite cell self-renewal (Figure 2.3) (Rudnicki et al., 2008).

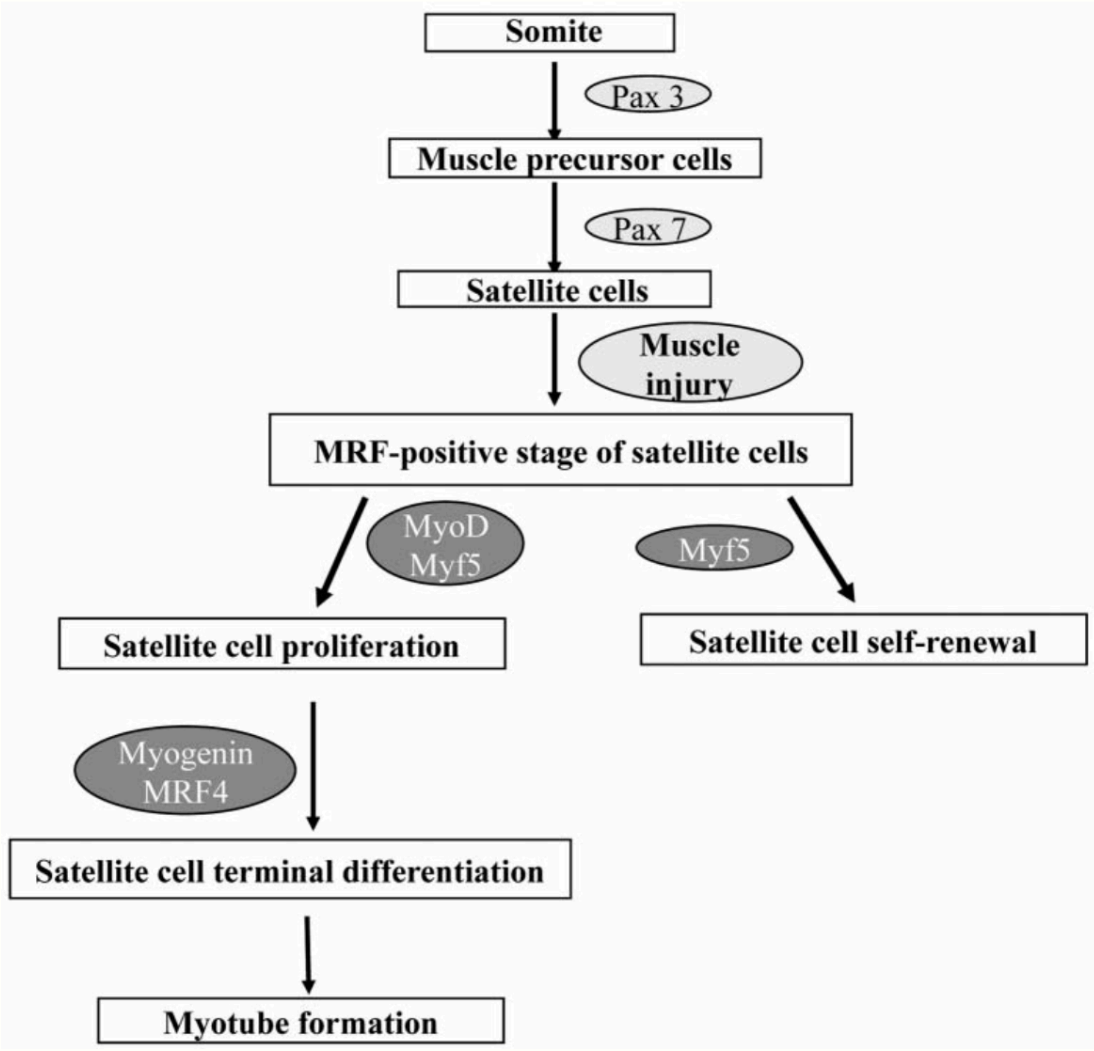

Figure 2.3. Satellite cell activation and differentiation in myogenesis and regeneration (Karalaki et al., 2009)

MyoD

MyoD is often considered to be the master regulatory gene in the myogenesis process, due to its ability to modify numerous cell types (fibroblasts, chondrocytes, neural cells) into myoblasts (Choi et al., 1990). It was the first discovered of the MRF family. Failure to express MyoD early in development leads to reduced differentiation and growth of muscles (Megeney, Kablar, Garrett, Anderson, & Rudnicki, 1996). Cultured MyoD-null myoblasts grow quickly, but abnormally express target muscle promoter genes (i.e., MCK, MyHC) and differentiate poorly (Sabourin, Girgis-Gabardo, Seale, Asakura, & Rudnicki, 1999). When MyoD genes were deleted in mouse models, Myf5 overexpressed and compensated for MyoD; despite vitality, muscle developed abnormally and showed defects in regeneration (Martin, 2003). In spite of Myf5's compensatory capacity, MyoD more effectively targets a greater number of genes inducing differentiation (Ishibashi, Perry, Asakura, & Rudnicki, 2005). Mice deficient in both MyoD and Myf5 fail to form skeletal muscle, and die at birth (Martin, 2003). MyoD mRNA has been shown to be more prevalent in fast glycolytic muscles and may play a role in fiber-type determination. Mice lacking functional MyoD genes shifted fiber type distribution and MyHC isoforms toward oxidative metabolism (Hughes, Koishi, Rudnicki, & Maggs, 1997).

MyoD appears to be explicitly involved in DDR processes of skeletal muscle. In response to DNA damage, an ABL (Abelson murine leukemia) tyrosine kinase phosphorylates MyoD at tyrosine 30, arresting myoblasts at the G_1/S checkpoint. MyoD is prevented from activating muscle gene expression for synthesis (Simonatto et al., 2011). Rather than performing transcriptional activation of muscle synthesis, MyoD

recruits phosphorylated Nbs1 (Nijmegen breakage syndrome 1 gene) to target and repair damaged chromatin. Nbs1 binds to phosphorylated H2AX foci of DSB and begins DNA repair activities (Kobayashi et al., 2004). MyoD-null mice fail to repair DNA lesions, allowing for proper synthesis activities to occur (Simonatto et al., 2013). When DNA damage is detected at the G_2/M phase checkpoint, MyoD cannot bind DNA sequences of target genes, and cell arrest is induced before mitosis occurs.

Myogenin

Myogenin is expressed at the entry into the terminal differentiation program, before fusion and differentiation of myoblasts to multinucleated myotubes (Smith, Janney, & Allen, 1994; Yutzey, Rhodes, & Konieczny, 1990). Knockout mouse models cause death at birth because myogenin$^{-/-}$ myoblasts are unable to fuse and form differentiated myofibers (Hasty et al., 1993; Rawls et al., 1998; Vivian, Olson, & Klein, 2000). Muscle progenitors in these myogenin -/- mice still express MyoD and Myf5, but muscle differentiation is defective (Martin, 2003). Unlike MyoD, myogenin transcript mRNA has been found to be expressed greater in slow MyHC isoforms (Hughes et al., 1993). Additionally, overexpression of myogenin results in greater oxidative metabolism, with a decrease in glycolytic enzyme concentration (Hughes, Chi, Lowry, & Gundersen, 1999). Myogenin has been shown to increase following aerobic exercise in oxidative muscle, such as the SOL (Siu et al., 2004).

Muscle Regulatory Factor 4

Muscle regulatory factor 4 (Mrf4) is expressed lastly during terminal differentiation contributing to cell maturation, but also plays a role in the commitment of cell to muscle lineage. Like Myf5, Mrf4 acts upstream of MyoD in determining skeletal

muscle identity in the early somite (Kassar-Duchossoy et al., 2004). Inhibition of Mrf4 results in reduced cell fusion and differentiation. $Mrf4^{-/-}$ models demonstrate severe muscle development deficiency. During early myogenesis, Mrf4 can act in place of myogenin and increase expression of Mrf4 genes and partial development of ribcage, but ultimately muscle development is defective and death occurs (Zhu & Miller, 1997). Mice deficient in both Mrf4 and MyoD display form similar to myogenin-null mice, with failure to differentiate myotubes (Rawls et al., 1998). Activities of Mrf4 are upregulated by MEF2/myogenin interaction, as well. Mrf4 is involved in the maintenance of muscle gene expression, and is present in mature myotubes. Expression of the other MRFs tend to decrease postnatally, but Mrf4 remains constant throughout the lifespan, as seen in mice (Bober et al., 1991; Hinterberger et al., 1991).

Myocyte Enhancer Factor 2

The myocyte enhancer factor 2 (MEF2) group of proteins are members of MADS (MCM1, agamous, deficiens, serum response factor) box-containing family of transcription factors. MEF2 was discovered as a protein from skeletal muscle nuclei, which bound an A/T-rich sequence in muscle creatine kinase (MCK) gene promoters (Gossett, Kelvin, Sternberg, & Olson, 1989). Structurally, MEF2 proteins have an amino terminal composed of MEF and MADS boxes, which dimerize and bind DNA, and a carboxyl terminal, involved in gene activation and kinase response (Black & Olson, 1998).

In vertebrates, MEF2 proteins act as co-factors for transcription during muscle development. Evidence suggests MEF2 synergistically activates gene expression after the onset of differentiation, implicating their role in later stages of terminal differentiation

(Naya & Olson, 1999). In transgenic mice and cultured cells, MEF2 proteins bind to and are necessary for the expression of myogenin and Mrf4 (Olson et al., 1995; Weintraub, 1993). Additionally, the myogenin and Mrf4 gene promoters do not have autoregulatory binding sites, but, in fact, have a site for the MEF2 protein, which enhances promoter activation (Edmondson, Cheng, Cserjesi, Chakraborty, & Olson, 1992; Naidu, Ludolph, To, Hinterberger, & Konieczny, 1995).

MEF2C genes are expressed in several tissues, but first appear in the precardiac mesoderm. Targeted inactivation of MEF2C in embryonic mice leads to lethal defects in cardiac development (Lin, Schwarz, Bucana, & Olson, 1997). Throughout the mouse embryo, MEF2C is also expressed in smooth muscles. MEF2A, -B, and -D are expressed ubiquitously in various non-muscle cells (Lyons, Micales, Schwarz, Martin, & Olson, 1995). A few hours after myogenin is expressed in skeletal muscle-bound cells, MEF2C becomes evident (Figure 2.4) (Edmondson et al., 1992). In adults, MEF2C expression is limited to skeletal muscle, brain, and spleen, but its roles are essential in the origins of cardiac and smooth muscle.

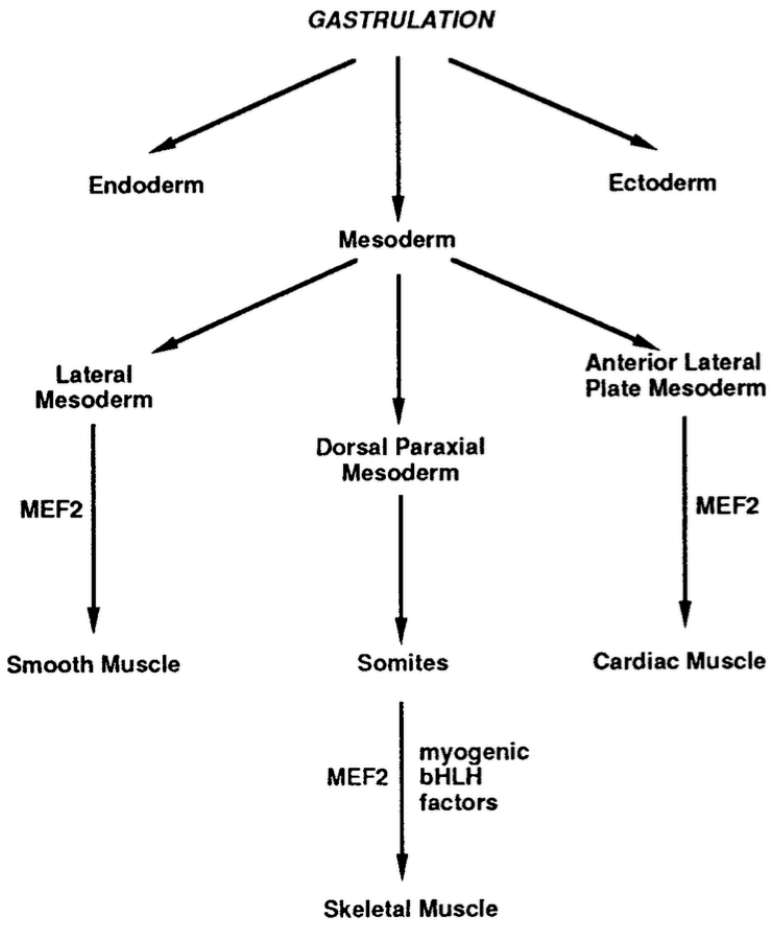

Figure 2.4. Sites of MEF2 proteins influence in myogenesis (Olson et al., 1995)

Inhibitor of DNA Binding

The inhibitor of DNA binding (Id) protein is an HLH (helix-loop-helix) structure that competitively binds to E-proteins. Id lacks the basic region for DNA binding seen in the bHLH family of MRFs. The binding affinity of Id is substantially greater than MRFs and acts as a negative regulator of the MRFs, sequestering potential of MRF dimer partners (Benezra, Davis, Lockshon, Turner, & Weintraub, 1990). In normal circumstances, Id is present and heterodimerizes with E-proteins, but the level expressed

is not adequate to bind all dimer partners. Remaining E-proteins are able to heterodimerize with MRFs and proceed in myoblast determination.

Id is expressed in a number of proliferating cells and typically decreases expression upon induced differentiation (Biggs, Murphy, & Israel, 1992; Sun, Copeland, Jenkins, & Baltimore, 1991). When levels increase substantially, the overexpression of Id in myoblasts inhibit muscle differentiation (Üyashi et al., 1994). Additionally, Id proteins are overexpressed in a nuÜber of human cancers and elevated expression is correlated with higher histological grades (Ruzinova & Benezra, 2003). Other factors such as Twist, Mist1, MyoR, and Sharp-1 also inhibit MRF transcriptional activities, DNA-binding, and E-protein dimerization (Azmi, Ozog, & Taneja, 2004; Lemercier, To, Carrasco, & Konieczny, 1998; Lu, Webb, Richardson, & Olson, 1999; Spicer, Rhee, Cheung, & Lassar, 1996).

Doxorubicin and Myogenic Regulatory Factors

C2C12 myoblasts exposed to DOX *in vitro* down-regulate MyoD and myogenin gene transcripts, while increasing levels of Id (Kurabayashi et al., 1993, 1994). As Id competes for E-protein binding, elevated expression reduces availability for MRF heterodimerization and transcript signaling for myoblast determination (Puri et al., 1997).

As previously mentioned, MyoD directly contributes to signaling of DNA repair when myoblasts are exposed to DNA-damaging agents (i.e., DOX). C2C12 myoblasts deficient in MyoD with inhibited ABL kinases exhibit impaired DNA repair activity when exposed to DOX compared to wild type (WT) controls. Removing ABL kinase inhibitors does not restore repair mechanisms, and requires functional MyoD to direct

phosphorylated Nbs1 to DNA-damaged foci on target genes, including myogenin promoter and muscle creatine kinase (MCK) enhancer (Simonatto et al., 2013).

DOX arresting myoblasts at the G_2/M phase does not allow MyoD to bind DNA and activate differentiation (Kurabayashi et al., 1994; Puri et al., 1997). DOX-induced arrest during the G_2 phase reduces MyoD occupancy on myogenin and MCK target genes (Simonatto et al., 2011). Puri and collegues (1997) exposed C2C12 mouse myoblasts to DOX, ceasing the cell cycle at the same checkpoint, and demonstrated DNA synthesis occurred without apoptosis following removal from DOX. These researchers found DOX treatment led to an up-regulation in p21 proteins. p21 is a cyclin D kinase inhibitor, which regulates passage through the cell cycle checkpoints. It responds to p53 by mediating cell cycle arrest without inducing apoptosis. After exposure to DOX, p21 expression positioned myotubes in a reversible arrested state (Puri et al., 1997). p53 either directly signals apoptosis or p21 to arrest cells in a senescent state for DNA repair before restarting the cell cycle.

Despite the shielded state of SCs to gentoxic stress while in a quiescent state, once activated for muscle regeneration, SCs become susceptible to DNA damage. SCs from MyoD-null mice exposed to DOX demonstrate DDR signaling; however, significant delay in response and incomplete DNA repair persists up to 48 hours following damage. Reintroduction of WT MyoD and ABL kinase restores repair ability and corrects DNA lesions in treated SCs (Simonatto et al., 2013).

The reduced expression of myogenic markers, MyoD and Myf5, suggests a decrease in the determination of satellite cells to become new muscle cells. An elevation in Myf5 expression has been shown to proliferate myoblasts, with some reversing into a

quiescent, satellite state for later differentiation. The increased SC number may assist with muscle regeneration in the period following DOX treatment after its systemic removal. The reduced expression of markers, Mrf4 and myogenin, will decrease the ability of potential myocytes to differentiate into myotubes. The α-like RNA polymerase core II subunit 3 (RPB3) specifically binds to myogenin, and not the other MRFs (Corbi et al., 2002). DOX down-regulates RPB3 expression, which is correlated with inhibited muscle differentiation. Activation of myogenin, and subsequent muscle differentiation, may be blocked due to reduced RPB3 via DOX treatment (Martin, 2003). Exposure of C2C12 myoblasts to genotoxic agents, such as DOX, reduced myotube formation by 60-75% and inhibited expression of myogenin and MyHC seen late in the myogenic process (Puri et al., 2002). Reduced levels of MyoD were not evident in this study, but E-box DNA binding by MyoD is dramatically reduced following DOX treatment in myocytes induced to differentiate (Puri et al., 1997).

As far as reductions in one MRF versus another, fluctuations can potentially affect the muscle's phenotype. In a normal, healthy state, oxidative (Type I) muscles express higher levels of myogenin, while fast, glycolytic (Type II) muscles express greater MyoD genes. Preserving myogenin in SOL and MyoD in EDL will presumably maintain the normal muscle status. With endurance TM training adaptations acting more upon oxidative muscles, increased/maintained muscle mass should be evident more so in these tissues.

Exercise Preconditioning

Just as muscle can hypertrophy due to exercise and growth factors, significant decreases in muscle size can occur as result of injury, disease, denervation, cachexia,

prolonged disuse, sepsis, and oxidative stress (Tisdale, 2009). Aerobic exercise training interventions are preventative measures used in mitigating the negative effects associated with cancer treatments (Dimeo, Fetscher, Lange, Mertelsmann, & Keul, 1997; Dimeo, Rumberger, & Keul, 1998; Chicco, Hydock, Schneider, & Hayward, 2006). Although acute exercise increases free radical generation and tissue damage, repetitive training appears to enhance antioxidant enzymes and oxidative repair systems (Gomez-Cabrera, Domenech, Ji, & Viña, 2006; Radak, Chung, & Goto, 2008; Radak, Chung, Koltai, Taylor, & Goto, 2008).

The effect of DOX generating uncontrollable ROS has been shown to decrease skeletal muscle mass and fatigue resistance. Increases in ROS scavenging antioxidants and attenuated functional deficits due to chronic aerobic exercise regimens prior to DOX administration are widely reported (Ascensão et al., 2012; Chicco, Hydock, Schneider, & Hayward, 2006; Chicco, Schneider, et al., 2006; Hydock et al., 2011b; Kanter, Hamlin, Unverferth, Davis, & Merola, 1985; Wonders, Hydock, Schneider, & Hayward, 2008). Short-term exercise protocols prior to DOX treatment have been shown to protect cardiac and skeletal muscle function and elevate antioxidant enzymes, as well (Gomez-Cabrera, Domenech, & Viña, 2008; Kavazis et al., 2014). Furthermore, acute aerobic exercise attenuates myofibrillar degradation of actin in skeletal muscle and prevents increases in apoptotic signaling following DOX i.p. injections (Smuder, Kavazis, Min, & Powers, 2011a; Smuder et al., 2011b). Even single bouts of endurance exercise have been shown to reduce lipid peroxidation seen in myocardial tissues following DOX injections (Wonders, Hydock, Schneider, & Hayward, 2008).

As previously mentioned, Mrf4 is expressed at higher levels in adult muscles than the other MRFs. Innervation is thought to alter the expression of these proteins, with higher levels expressed following physical activity. Interestingly, with muscle denervation, Myf5 and MyoD mRNA levels increase at least two-fold, whereas myogenin transcript levels are elevated dramatically (Neville, Schmidt, & Schmidt, 1992; Voytik, Przyborski, Badylak, & Konieczny, 1993). When re-innervated, however, transcript levels rapidly return to basal conditions, without altering Mrf4 expression. Mice subjected to immobilization (muscle disuse) had increased levels of myogenin mRNA in the fast-twitch plantaris muscle while in lengthened positions. Shortened positions decreased muscle mass and Mrf4 mRNA levels in the slow-twitch soleus (SOL) muscle (Loughna & Brownson, 1996). Despite increases of mRNA following denervation and immobilization, functional protein expression has not shown similar effects. Effective protein expression appears to actually influence muscle mass versus mRNA, with the exception of Mrf4 in the shortened position of the SOL muscle.

Neuromuscular activity, such as exercise, may influence functional MRF protein expression in a more substantive manner. Siu and collegues (2004) found 24% greater myogenin protein expression in rats following 8 weeks of treadmill (TM) training than that of controls. Furthermore, oxidative metabolic enzyme levels and activity (i.e., citrate synthase, cytochrome-c oxidases) exhibited linear increases, which may assist in handling of ROS. As myogenin is a secondary MRF, its expression may be more readily examined following acute exercise training. With single bouts of resistance exercise in humans, MRF mRNA expression increases, but returns to basal levels within 24 hours (Psilander, Damsgaard, & Pilegaard, 2003; Yang, Creer, Jemiolo, & Trappe, 2005). Haddad and

Adams (2002) found greater myogenin transcript levels in rats with repeated bouts of exercise. Repeated endurance exercise bouts may elicit similar elevations in functional MRF protein levels. MRF transcript levels are found to decay 72 hours following exercise bouts (Bickel et al., 2005). Kosek and colleagues (2006) investigated responses to acute and chronic resistance training in humans. Following a single bout, no significant changes occurred in protein expression, whereas mRNA increased in myogenin alone. Following sixteen weeks of training, transcript levels of Myf5, MyoD, and myogenin were increased from baseline. Protein levels Mrf4 and myogenin were significantly higher after chronic resistance exercise (Kosek et al., 2006). No intermediate measurements were recorded to distinguish transitions from MRF mRNA to protein synthesis. Potential elevations of Myf5 and MyoD proteins may return to basal levels after chronic training, which might be revealed following short-term exercise training.

MyoD has been implicated as a key regulator of myogenic transcription, due to its association with ABL tyrosine kinase and ability to destabilize the non-functional DNA-TOP2 complex. If levels of MyoD are up-regulated/maintained with short-term exercise training, muscle regeneration may be more viable following DOX treatment. Siu and collegues (2004) did not observe significant MyoD increases in SOL muscles following a TM protocol, but had the EDL been examined, levels may have been elevated due to fiber type and MRF expression patterns (Hughes et al., 1997; Hughes et al., 1993; Siu et al., 2004).

Conclusion

Skeletal muscle cells' ability to regenerate is impaired following exposure to DOX. p53-dependent apoptotic programs inhibit mitotic divisions and differentiation of

new myocytes. Additionally, DOX produces DNA lesions and prevents repair due to its inhibition of TOP2 function. Impaired ability to counter DNA damage in skeletal muscle could lead to accumulated lesions and loss of muscle integrity over time. Chronically elevated ROS production in skeletal muscle leads to proteolysis and cell death (Ji, Gomez-Cabrera, & Vina, 2006).

Aerobic exercise enhances ROS handling in skeletal muscle and lessens cellular damage attributed to oxidative stress. Additionally, aerobic training regimens will theoretically maintain the expression of MyoD and other MRFs, increasing the ability to maintain skeletal muscle DNA integrity following acute DOX treatment. DOX treatment elevates Id protein expression and decreases muscle force production *ex vivo*. Preconditioning, however, should potentially attenuate these DOX-induced dysfunctions in skeletal muscle.

Much of the existing literature focuses on *in vitro* myoblast exposure to DOX. An *in vivo* investigation may elucidate dynamic responses of muscle regeneration and functional MRF expression with prior aerobic conditioning. This study potentially demonstrated the therapeutic advantage of a short-term aerobic exercise intervention before DOX treatment to preserve skeletal muscle and future developmental capacity. Given the overlapping behaviors of MRFs, preservation of one or all should enhance muscle regenerative capacity, or temper muscle loss, and attenuate functional reductions following DOX treatment.

CHAPTER III

METHODOLOGY

Experimental Design

Ten-week-old male Sprague Dawley rats (N=47) were randomly assigned to either treadmill training (EXER, n=27) or sedentary (SED, n=20) groups. After 2 weeks of endurance training, animals were randomly assigned to receive either a DOX or placebo injection. Animals received either 15 mg/kg DOX or saline (SAL) injection (Figure 3.1). Skeletal muscle function was measured *ex vivo* 24 hours post DOX/SAL injections. A lipid peroxidation assay was used to determine levels of oxidative stress. Western blot analysis was used to quantify the myogenic regulatory factor proteins, Myf5, MyoD, myogenin and Mrf4, in SOL and EDL.

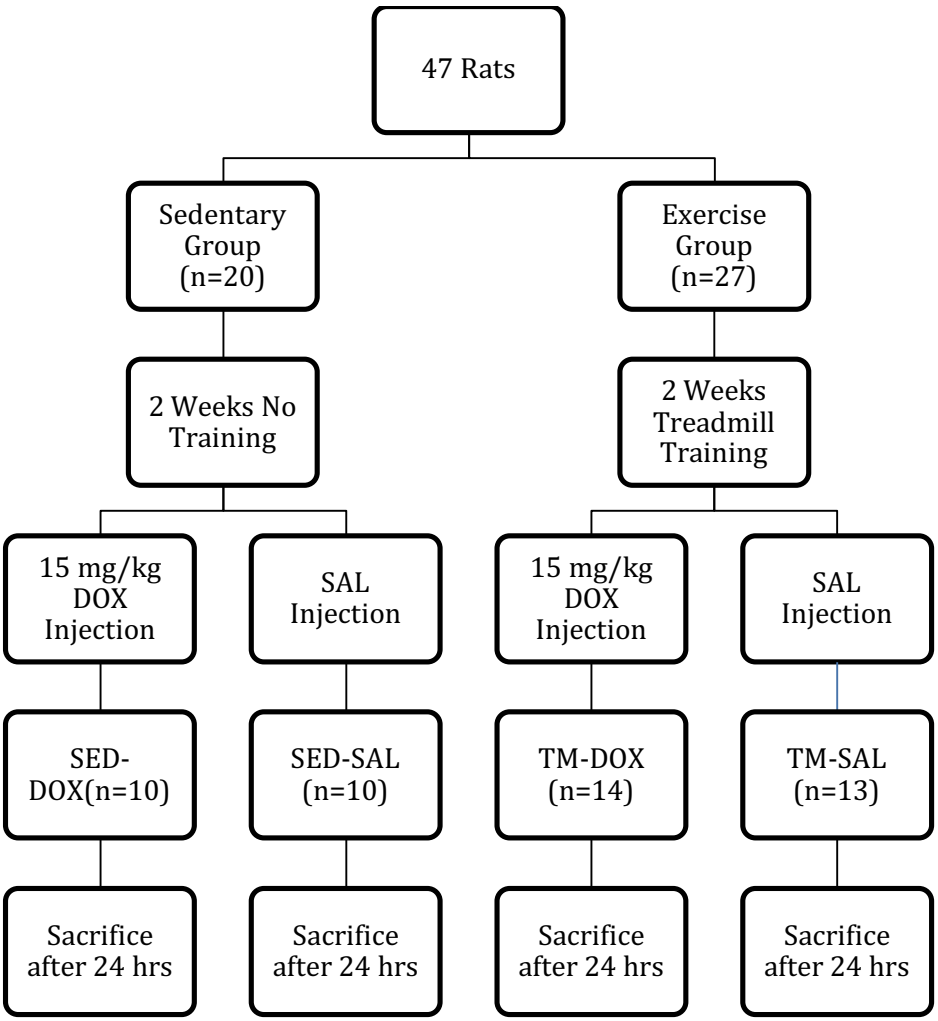

Figure 3.1. Experimental Design for Treatments

Animals and Animal Care

All experimental procedures were approved by the University of Northern Colorado Institutional Animal Care and Use Committee (Protocol #1407C-DH-R-17) and were in compliance with the Animal Welfare Act guidelines (see Appendix A). Male Sprague Dawley rats (10-week old) were purchased from Harlan (Indianapolis, IN). All rats were housed in pairs under a 12:12 light-dark cycle at room temperature (20 ± 2°C),

and food (Harlan Teklad 2016 rodent chow) and water was provided *ad libitum*. Rats were randomly assigned to one of four groups: (1) SED-SAL (Sedentary, Saline; n=10), (2) SED-DOX (Sedentary, DOX; n=10) (3) EXER-SAL (Exercise, Saline; n=13) and (4) EXER-DOX (Exercise, DOX; n=14). Body mass (g) for each animal was obtained at the start and end of training and before sacrifice.

Exercise Training Protocol

Animals assigned to exercise preconditioning groups trained for 2 weeks on a motorized treadmill during their dark cycle. In week 1, animals began running on a treadmill for 10 minutes and duration was increased 10 minutes each day. During week 2, animals trained for 60 minutes total per session (see Table 3.1). Speed remained constant at 30 meters per minute. The treadmill work rate used represents an estimated 70% of VO_2max (Lawler, Powers, Hammeren, & Martin, 1993). When necessary, rats were motivated by light electric shock at the rear of treadmill lanes. An exercise regimen of similar conditions provoked significantly less ROS generation in rats that trained on treadmills compared to sedentary animals (Smuder et al., 2011b). Furthermore, human experiments demonstrated the need for more than one exercise bout to prompt myogenin-positive stained satellite cells, and thus, differentiating myoblasts (Raue, Slivka, Jemiolo, Hollon, & Trappe, 2006). All animals assigned to EXER completed training protocols.

Table 3.1

Treadmill exercise training protocol

Day	1	2	3	4	5	6 – 7	8 - 12
Speed (m/min)	30	30	30	30	30	(Rest)	30
Duration (min)	10	20	30	40	50	(Rest)	60
Incline (%)	0	0	0	0	0	(Rest)	0

Drug Treatment

To eliminate acute effects of exercise, animals received injections 24 hours after the completion of the last treadmill training session. One half of both sedentary (10) and exercise (14) trained groups were randomly assigned to receive DOX hydrochloride (Bedford Labs: Bedford, OH) injections. The remaining animals (23) received 0.9% saline placebo. A bolus injection of 15 mg/kg DOX was delivered intraperitonally (i.p) on the right side of the abdominal cavity. Control animals received an equivalent volume of saline. Twenty-four hours after injections, rats were sacrificed and tissues extracted. The period between exercise and injection allowed sufficient time for MRF protein synthesis. Additionally, the twenty-four hour time period minimized effects due to anorexia-associated catabolism following treatment. Animals treated with DOX significantly reduce food and water intake within 24 hours to several days (Gilliam et al., 2009). Muscle masses should not be significantly affected in this 1-day period.

Isolated Muscle Function

Tissue Preparation

Animals were acutely anesthetized with sodium pentobarbital (50 mg/kg). After animals were completely anesthetized, indicated by failure to respond to a tail pinch, the soleus (SOL) and extensor digitorum longus (EDL) were be quickly excised from the right hindlimb and transferred to a tissue bath of Krebs Henseleit buffer (120 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 MgCl, 25 $NaHCO_3$, 17 glucose; in mM) for muscle function data collection. Animals were sacrificed prior to recovery from anesthesia by removal of the heart. Contralateral muscles were also be removed, trimmed free of connective tissue and fat, blotted dry, weighed, flash frozen in liquid nitrogen and stored at -80°C for later biochemical analysis.

The SOL and EDL muscles were chosen for examination to give a representation of two different muscle types (Type I: SOL; Type 2: EDL). Muscles were removed 1 day following the end of training because oxidative enzymes are not affected more than 48 hours after acute endurance exercise training (Siu, Donley, Bryner, & Alway, 2003). In young humans, MRF mRNA levels peak 12 hours after exercise (Williamson, Godard, Porter, Costill, & Trappe, 2000). mRNA transcripts, however, are not functional proteins. In fact, MRF mRNA can be upregulated without activation of SCs (Lowe & Alway, 1999). MyoD and myogenin have been shown in myonuclei as early as 1 day post functional overload in rodent models (Ishido, Kami, & Masuhara, 2004).

Ex Vivo Muscle Function

Functional muscle data were obtained via electrostimulation of tissues in organ baths (Radnoti: Monrovia, CA). Two electrodes surrounding the muscle in organ baths provided stimulation. Muscle contraction forces were recorded using PowerLab data acquisition hardware (ADInstruments: Colorado Springs, CO). Maximal twitch force was achieved by adjusting muscle for optimal length, and subsequent change in voltage applied (Grass Technologies: Warwick, RI).

The muscle stimulation methods follows the protocol reported by Hydock et al. (2011). Muscles were allowed to stabilize in the warmed (37°C) and oxygenated (95% O_2/5% CO_2) organ bath prior to functional data collection. Initial muscle tension was adjusted to 0.5 g, and stimulated with a square-wave pulse duration of 0.5 ms at 40 V. Muscle tension was increased by 0.2 g per stimulation (2-minute rest) until twitch force reaches a maximum. Next optimal voltage was verified by increasing applied voltage by increments of 5 V, allowing the same 2-minute rest period. Once maximal twitch force was determined, the bath of Krebs-Henseleit buffer was be cycled for a new volume to ensure proper electrolyte balance for ensuing fatigue resistance recording. The muscle was allowed a recovery time of 30 minutes in fresh buffer before continuous stimulation cycling. Pulse duration was increased to 500 ms while maintaining the determined optimal length and voltage. The muscle was stimulated continuously every 1 second for 2 minutes to simulate fatiguing conditions (Hydock, Lien, Jensen, Schneider, & Hayward, 2011a). LabChart software (ADInstruments) was used to analyze force data acquired.

Measures of maximal force, maximal rate of force development, and maximal rate of force decline were recorded during the single twitch force stimulation recordings.

During the continuous, fatiguing protocol, force production were recorded in reference to baseline levels every 10 seconds, for a total of 100 seconds.

Biochemical Analyses

Homogenate Preparation

The flash frozen left hindlimb muscles were homogenized in radioimmunoprecipitation assay (RIPA) buffer (10:1) and protease enzyme inhibitors (SigmaAldrich: St. Louis, MO). After manual homogenization, samples were sonicated to increase nuclear protein recovery. Homogenates were then be spun in a microcentrifuge for 10 minutes at 3000 *g* at 4°C. Total protein was quantified in samples using a Genesys 20 photospectrometer (ThermoSpectronic: Rochester, NY) at 562 nm according to the bicinchronic acid (BCA) assay (Smith et al., 1985). A determined amount of RIPA buffer was added to standardize protein concentration.

Lipid Peroxidation

The most abundant product of LPO is malondialdehyde (MDA), and it is commonly assayed as an index of oxidative stress. LPO was determined using a commercially available assay kit (BioxyTech MDA-586, Oxis Research: Foster City, CA). Malondialdehyde and 4-hydroxyalkenals (MDA+4-HAE) were measured to indicate cellular lipid peroxidation. 200 µL of sample homogenates were added to a microcentrifuge tube. 650 µL of N-methyl-2-phenylindole in acetonitrile was added to the sample and briefly vortexed. 150 µL of methanesulfonic acid was then be added and vortexed, followed by a 60 minute incubation period at 45°C. Following the incubation period, samples were centrifuged at 10,000 *g* for 10 minutes to remove turbidity. Supernatants were then transferred to cuvettes for absorbency measurement using a

spectrophotometer at 586 nm. Concentration of MDA+4-HAE was estimated from a standard curve. Samples were run in duplicate, with reassay if samples vary by more than 5%.

Western Blotting

Western blot analysis was conducted on muscle samples for the presence of MRFs in the SOL and EDL via SDS-PAGE. An equal volume of Lammeli buffer was added to samples in a microcentrifuge polypropylene vial, reducing protein concentration in half. Samples were heated in boiling water for 2 minutes, then chilled on ice for 5 minutes before 46 μg was loaded onto 4-20% gradient Tris-Glycine NuPage precast gels (LifeTechnologies: Carlsbad, CA). Gels were run at 125 V (constant voltage) and 4 mA current for 2 hours in a Xcell II blot module (Invitrogen, LifeTechnologies), until proteins had migrated the whole gel. Proteins were transferred to 0.45 micron polyvinylidene fluoride (PVDF) membranes over 90 minutes at 25 V and 100 mA. Protein transfers to PVDF membranes were ensured by the presence of a SeeBlue® Plus2 protein ladder (Novex, LifeTechnologies). Membranes were blocked for 1 hour in 15 mL of TBST + 5% milk, and then incubated with gentle agitation overnight in 10 mL of primary antibodies. Membranes were then be washed in TBST three times for 5 minutes, followed by incubation in appropriate species-specific secondary antibodies for 1 hour. After three more 5-minute washes in TBST, membranes were prepared for protein band detection.

Detection was executed by enhanced chemiluminescence (ECL) (C-Digit, Li-Cor: Lincoln, NE), and ImagJ software (NIH: Bethesda, MD) was used to quantify protein expression. Immediately before chemiluminescent imaging, 1.5 mL of luminol and

enhancer (SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate, ThermoScientific: Waltham, MA) was added to the membrane. The primary antibodies of interest included rabbit monoclonal MyoD and myogenin (Santa Cruz Biotechnology: Dallas, TX) and Myf5 and Mrf4 (Abcam: Cambridge, MA). The rabbit monoclonal anti-GAPDH (Abcam) was used as a loading control. Molecular weights of protein bands were ensured in reference to a MagicMark™ XP standard ladder (Novex, LifeTechnologies). Secondary antibodies (Santa Cruz Biotechnology) corresponded to associated species (rabbit) and include horseradish peroxidase (HRP) for adequate reactivity.

Statistical Analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad: LaJolla, CA) and presented as means ± standard error (mean±SEM). A two-factor (Exercise X Drug) analysis of variance (ANOVA) was used to determine main effects and interactions of treatments in muscle mass, muscle force production, lipid peroxidation, and MRF concentration. If a significant F-value was observed, a Tukey *post-hoc* pair-wise comparison identified significant differences between groups. Variables included the four MRF proteins concentrations, muscle masses, and force parameters from *ex vivo* analysis in each of the groups (maximal twitch, maximal rate of force production, and maximal rate of force decline). Muscle fatigue responses were analyzed with maximal twitch force obtained every 10 seconds and compared to baseline measures. For this study, muscle fatigue was defined at the point at which force production was below 75% of baseline skeletal muscle twitch force. For statistical procedures, significance was set at the $\alpha=0.05$ level.

CHAPTER IV

RESULTS

The purpose of this study was to determine the effects of acute DOX administration on skeletal muscle force production and fatigue resistance, lipid peroxidation (LPO), and MRF expression in skeletal muscles. Additionally, this study investigated the effects of short-term endurance exercise training on muscle force production and fatigue resistance, LPO, and MRF expression. This chapter presents findings of the study.

General Observations

Table 4.1 presents animal characteristics at the time of injection after training and at the time of sacrifice. At the time of injection, animal body mass was significantly different between groups. An activity effect was observed with SED animals exhibiting significantly greater body mass, F (1, 43) = 16.72, $p<0.05$. *Post hoc* testing revealed body mass to be higher in SED-SAL and SED-DOX than EXER-SAL, $p<0.05$.

Table 4.1

Animal Characteristics.

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Injection Body Mass (g)	360 ± 11 [a]	364 ± 10 [a]	322 ± 5	335 ± 7
Sacrifice Body Mass (g)	360 ± 12 [b]	354 ± 9 [b]	326 ± 6	329 ± 6
SOL Mass (mg)	135 ± 7	140 ± 4	132 ± 5	133 ± 5
EDL Mass (mg)	142 ± 5	138 ± 8	135 ± 4	141 ± 4

SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM.
[a] = Significantly greater than EXER-SAL ($p<0.05$).
[b] = Significantly greater than EXER-DOX ($p<0.05$).
Significant activity effect in injection and sacrifice body mass ($p<0.05$).

At the time of sacrifice, a similar activity effect existed with greater mass in SED animals, F (1, 43) = 13.42, $p<0.05$. *Post hoc* testing revealed that EXER-DOX body mass was significantly less than SED-SAL and SED-DOX, $p<0.05$. A significant drug effect was observed in body mass change, as well, F (1.43) = 34.65, $p<0.05$ (Figure 4.1). Rats receiving SAL slightly gained body mass after injections (1%) while those receiving DOX decreased body mass (2.5%). Within groups, body mass changes were not significantly different ($p>0.05$).

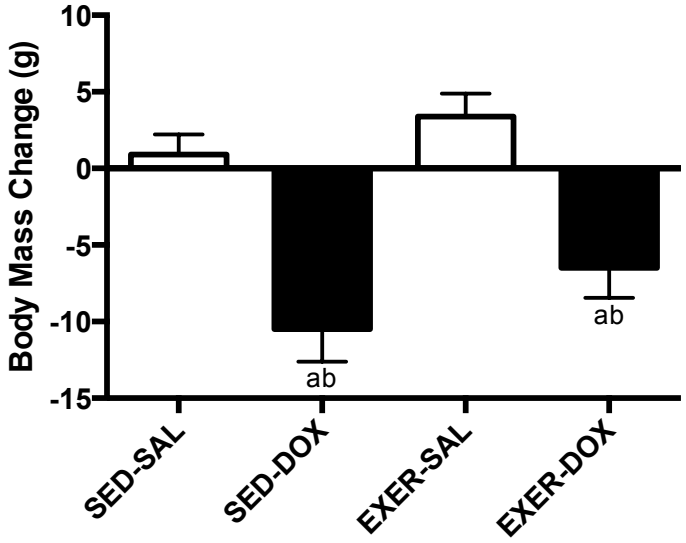

Figure 4.1. Change in body mass following injections.
SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL ($p<0.05$).
[b] = Significantly different from SED-SAL ($p<0.05$).
Significant drug effect ($p<0.05$).

There was no significant difference in absolute SOL mass observed between groups, $p>0.05$ (Figure 4.2A). Similarly, when SOL mass was corrected for body mass, no significant difference was detected, $p>0.05$ (Table 4.2, Figure 4.3A). Absolute EDL masses displayed no significant differences for main effects or exercise by drug interaction, $p>0.05$ (Figure 4.2B). When corrected for body mass, an activity effect was detected with endurance-trained animals demonstrating higher relative EDL mass, $F = (1, 43) = 4.871$, $p<0.05$ (Figure 4.3B).

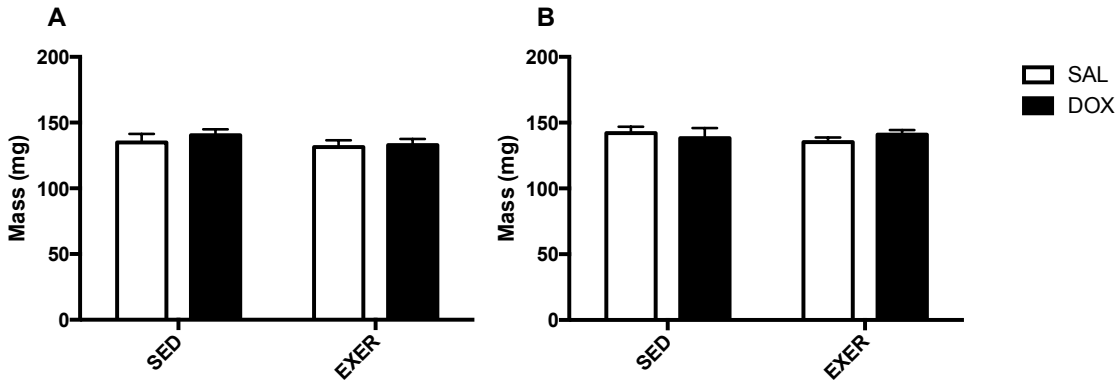

Figure 4.2. Tissue mass at time of sacrifice, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Table 4.2

Tissue mass relative to body mass.

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
SOL (mg/g BM)	0.37 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.41 ± 0.01
EDL (mg/g BM)	0.39 ± 0.01	0.39 ± 0.02	0.42 ± 0.01	0.43 ± 0.01

SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant activity effect in EDL ($p<0.05$).

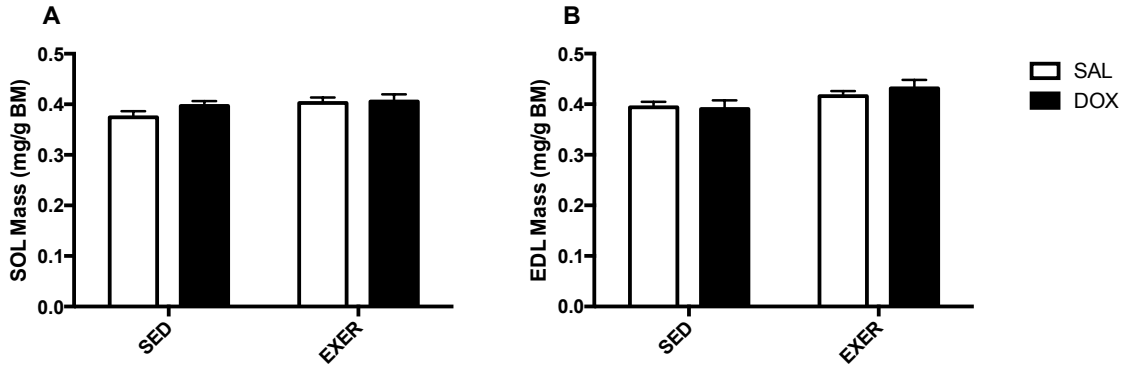

Figure 4.3. Tissue mass relative to body mass, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, BM = body mass, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant activity effect in EDL ($p<0.05$).

Isolated Muscle Function

Maximal Twitch Force

Once right SOL and EDL were excised, muscles were placed in organ baths filled with Krebs-Henseleit buffer. Tension and voltage were gradually increased to determine optimum conditions as detected by maximal twitch force elicited. Additionally, rate of force production and rate of force decline were measured from maximal twitch force tracings.

No significant differences were observed in SOL maximal twitch forces between groups, $p>0.05$ (Table 4.3). After adjusting force relative to tissue mass, significance remained undetected (Figure 4.4). Rates of force production and decline were also not significantly different between groups, $p>0.05$.

Table 4.3

SOL muscle twitch forces

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Maximal Twitch Force (mN)	37 ± 3	50 ± 5	44 ± 4	46 ± 4
Relative Maximal Twitch Force (mN/g)	249 ± 36	287 ± 29	258 ± 28	296 ± 41
Maximal Rate of Force Production (mN/s)	4916 ± 463	6376 ± 699	5478 ± 670	5536 ± 589
Maximal Rate of Force Decline (mN/s)	- 1715 ± 193	- 1935 ± 160	- 2027 ± 117	- 2255 ± 154

SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

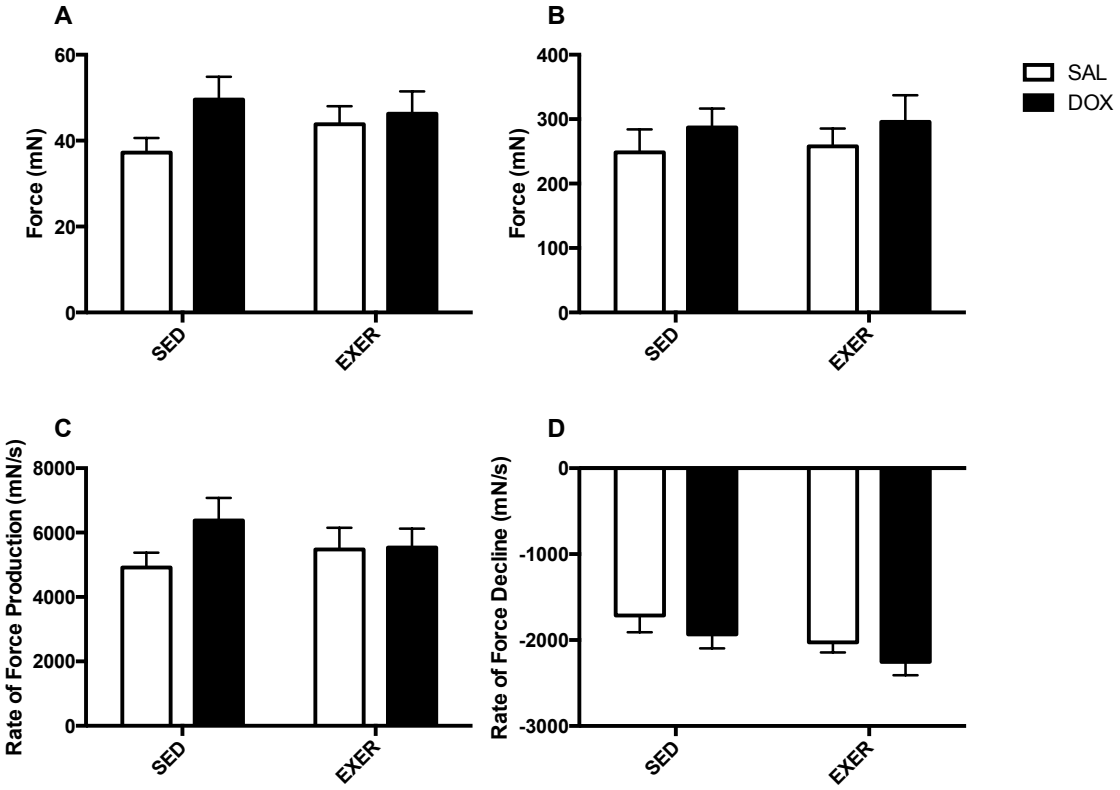

Figure 4.4. SOL Maximal twitch force values. Maximal twitch (A), maximal twitch relative to tissue mass (B), rate of force production (C), rate of force production (D), SOL rate of force decline.
SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

No significant differences were seen between groups in EDL maximal twitch forces, $p>0.05$ (Table 4.4). Similarly, no significance was observed after correcting for tissue mass. Rates of force production and decline were not significantly different between groups, as well (Figure 4.5).

Table 4.4

EDL muscle twitch forces

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Maximal Twitch Force (mN)	79 ± 7	90 ± 9	92 ± 8	77 ± 12
Relative Maximal Twitch Force (mN/g)	511 ± 26	603 ± 69	632 ± 51	545 ± 80
Maximal Rate of Force Production (mN/s)	14407 ± 1199	17851 ± 2060	18242 ± 1772	14966 ± 2603
Maximal Rate of Force Decline (mN/s)	- 8489 ± 690	- 10410 ± 1155	- 11583 ± 1272	- 9308 ± 1380

EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

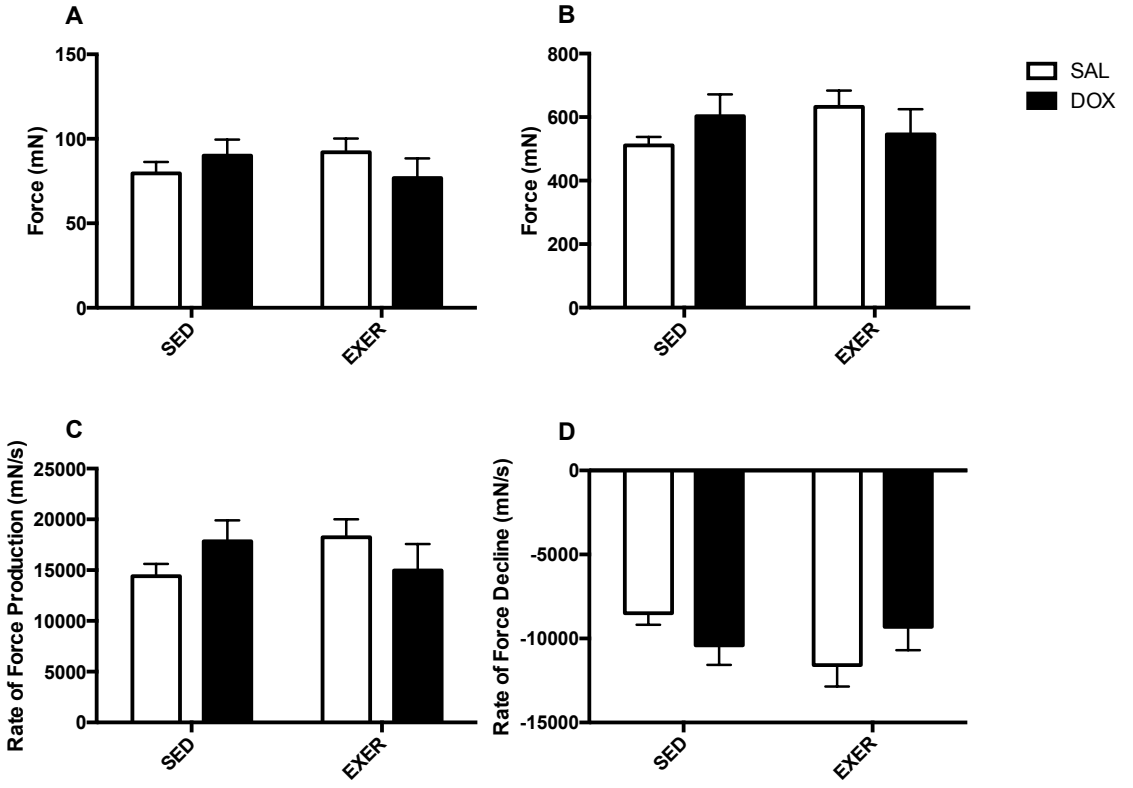

Figure 4.5. EDL Maximal twitch force values. Maximal twitch (A), maximal twitch relative to tissue mass (B), rate of force production (C), rate of force production (D), SOL rate of force decline.
EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Fatigue

After cycling Krebs-Henseleit buffer and allowing muscles to rest for 30 minutes, a 2-minute continuous stimulation at determined optimal length and voltage was performed. Time-to-fatigue was determined when muscles produced 75% of baseline force production.

In the SOL, SED-DOX and EXER-SAL generated less than 75% baseline force 40 seconds into fatiguing protocol. At the 50-second time point, EXER-DOX force

production was below 75%. SED-SAL recorded the greatest fatigue resistance with twitch forces lower than 75% of baseline by 70 seconds (Figure 4.6).

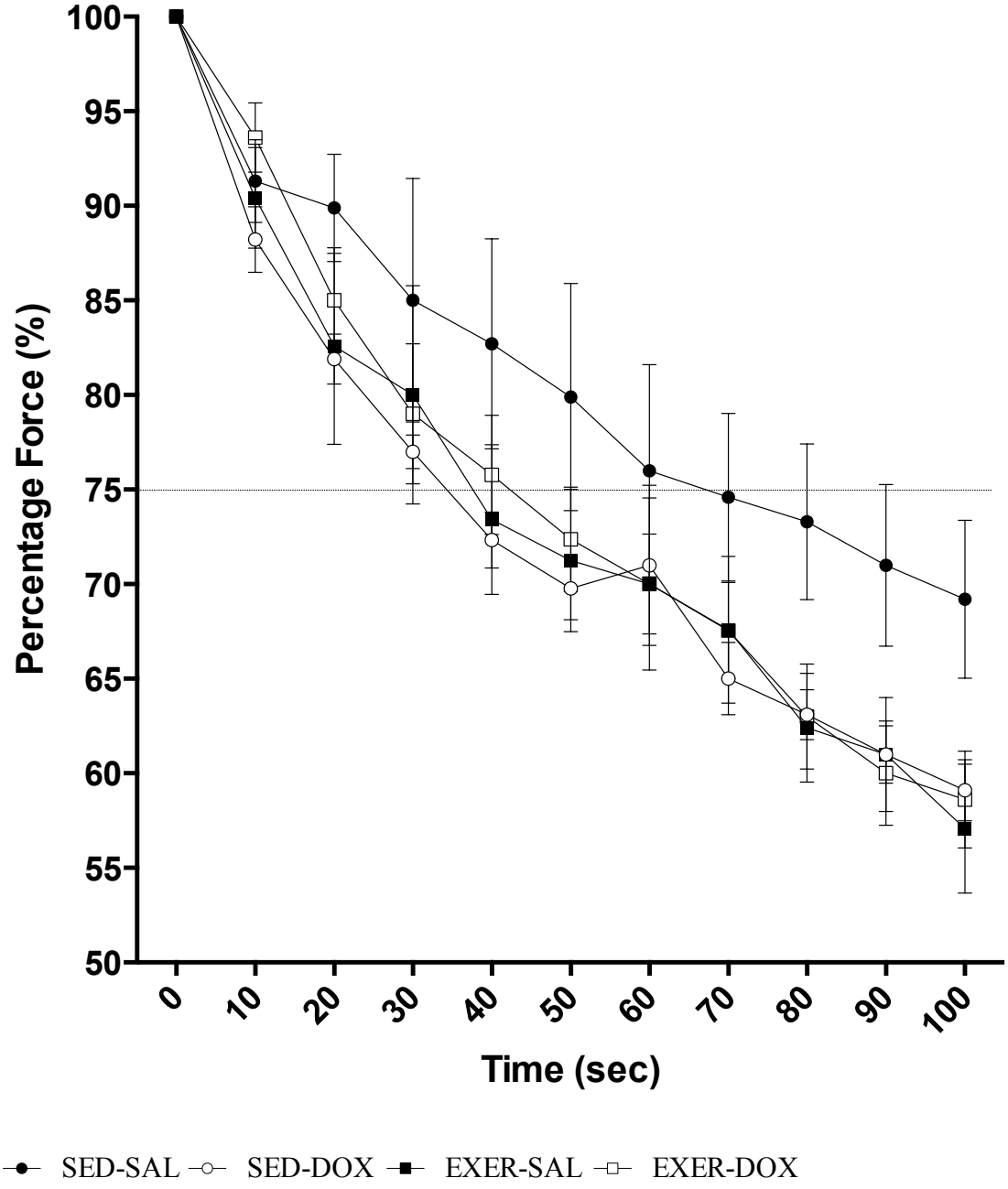

Figure 4.6. SOL fatigue resistance.
SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM.

In the EDL, SED-SAL fatigued quickest with twitch forces less than 75% at the 40-second time point. SED-DOX, EXER-SAL, and EXER-DOX groups' forces fell below fatigue threshold at 50 seconds (Figure 4.7). The fast-twitch muscle typically fatigues a greater rate than slow, oxidative muscles. *Ex vivo* force production of the EDL did not fatigue faster to levels below 75% of initial force values but, at the end 100 seconds, all forces were below those of SOL.

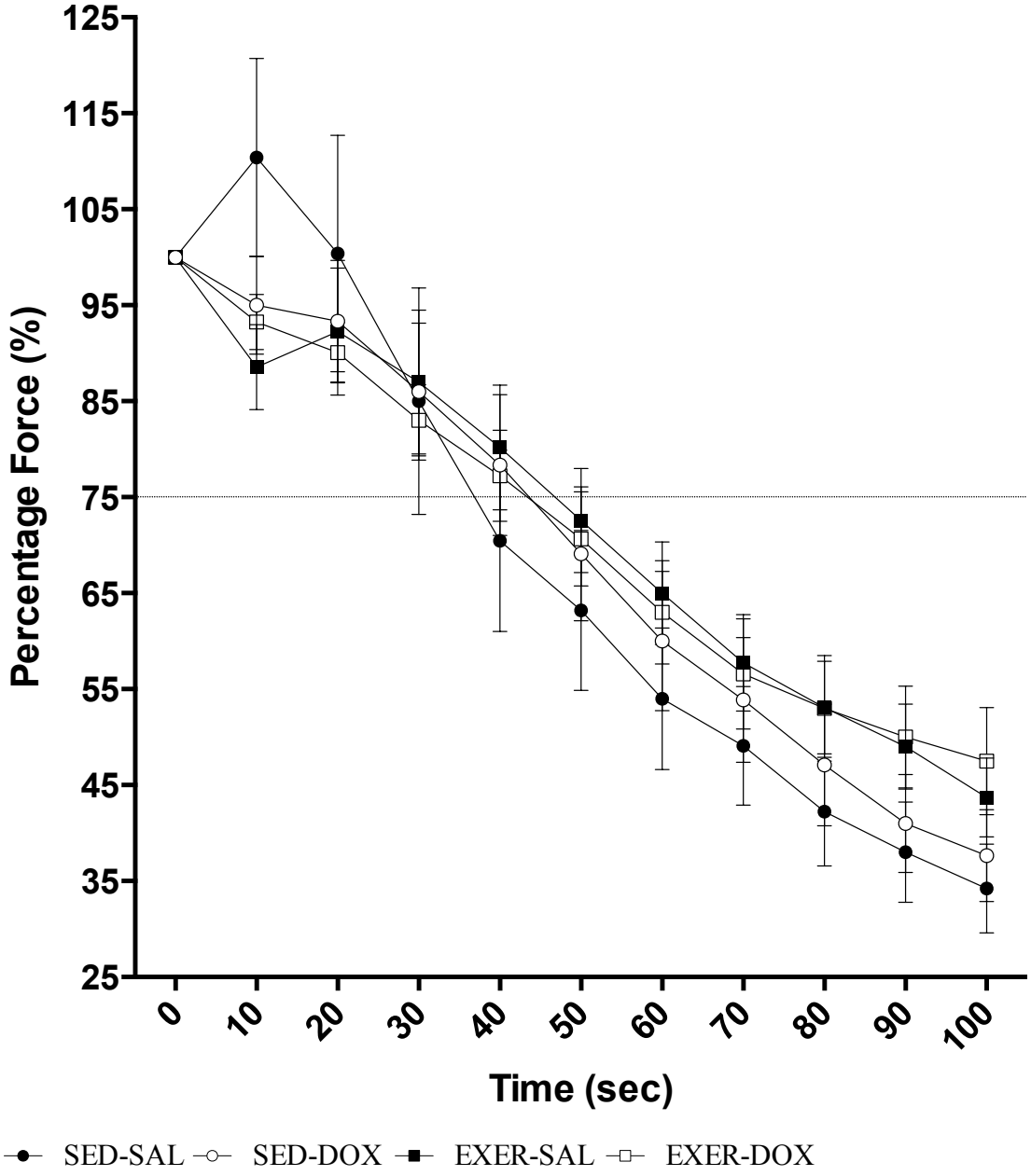

Figure 4.7. EDL fatigue resistance.
EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM

Biochemical Analyses

Lipid Peroxidation

SOL and EDL homogenates obtained from rats 1 day after saline or DOX injection were analyzed for markers of lipid peroxidation (MDA+4-HAE). All data from LPO analysis are presented in Table 4.5. In SOL, a significant drug effect was observed in rats receiving SAL exhibited higher levels of MDA+4-HAE, $F = (1, 43)$, $p<0.05$ (Figure 4.8A). EXER-SAL animals presented significantly greater indices of lipid peroxidation than SED-DOX and EXER-DOX. No activity effect or interaction was detected in the SOL. In the EDL, a significant activity effect was observed with TM animals presenting higher MDA+4-HAE, $F = (1, 43) = 4.08$, $p<0.05$ (Figure 4.8B). No drug effect or interaction was detected.

Table 4.5

Lipid peroxidation levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
SOL (pmol/mg)	329 ± 24	275 ± 17 [a]	398 ± 36	292 ± 17 [a]
EDL (pmol/mg)	214 ± 14	225 ± 17	283 ± 28	288 ± 44

SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL ($p<0.05$).
Significant drug effect in SOL.
Significant activity effect in EDL.

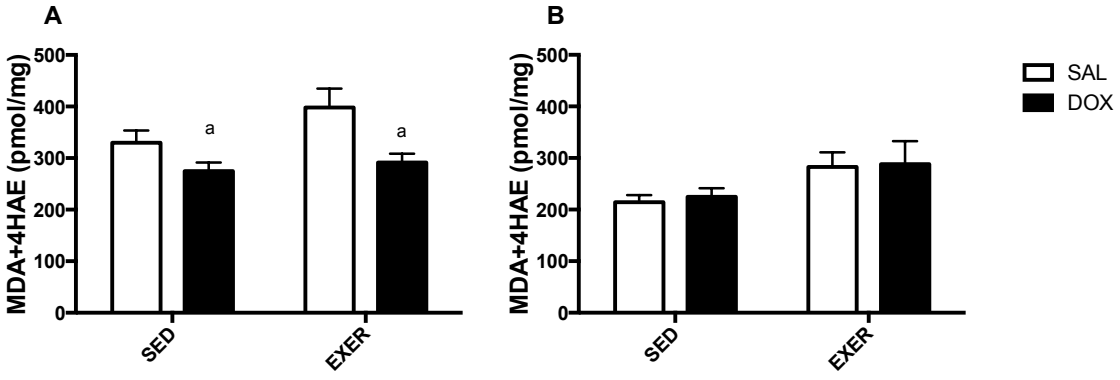

Figure 4.8. Lipid peroxidation levels in hindlimb muscles, SOL (A) and EDL (B). SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL.
Significant drug effect in SOL (p=0.0033).
Significant activity effect in EDL (p<0.05).

Western Blotting

Expression of myogenic regulatory factor proteins, Myf5, MyoD, Mrf4, and myogenin, were measured in SOL and EDL homogenates to evaluate the influence of endurance exercise and DOX on these transcription factors. Forty-six μg of protein from SOL and EDL homogenates were added to 4-20% Tris-glycine precast gels and run through SDS-PAGE. MRF levels were assessed by chemiluminescence and expressed relative to GAPDH as a loading control. It should be noted that no significant GAPDH activity of drug main effects or interactions (p>0.05) were observed suggesting that the exercise or drug treatments did not affect the loading control. All Western blot data are presented in Tables 4.6 (SOL) and 4.7 (EDL).

Table 4.6

SOL Myogenic Regulatory Factor (MRF) levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Myogenic Factor 5 (Myf5)	0.44 ± 0.10 [a]	0.73 ± 0.14	0.92 ± 0.08	1.28 ± 0.22
MyoD	1.29 ± 0.13	1.67 ± 0.13	1.46 ± 0.16	2.11 ± 0.31
Myogenin	0.50 ± 0.12	0.67 ± 0.14	0.81 ± 0.17	0.80 ± 0.16
Muscle Regulatory Factor 4 (Mrf4)	0.62 ± 0.18	0.80 ± 0.20	1.23 ± 0.18	1.22 ± 0.24

SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-DOX ($p<0.05$).
Significant activity and drug effect in Myf5 ($p<0.05$).
Significant drug effect in MyoD ($p<0.05$).
Significant activity effect in Mrf4 ($p<0.05$).

Table 4.7

EDL Myogenic Regulatory Factor (MRF) levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Myogenic Factor 5 (Myf5)	1.25 ± 0.23	1.25 ± 0.19	1.57 ± 0.19	1.49 ± 0.14
MyoD	1.28 ± 0.20	1.55 ± 0.17	1.30 ± 0.07	1.13 ± 0.09
Myogenin	0.48 ± 0.15	0.64 ± 0.15	0.62 ± 0.11	0.67 ± 0.14
Muscle Regulatory Factor 4 (Mrf4)	0.47 ± 0.12	0.52 ± 0.13	0.64 ± 0.09	0.46 ± 0.08

EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Myogenic Factor 5

In the SOL, a 2-way ANOVA revealed a significant activity effect, with EXER groups exhibiting higher levels of myogenic factor 5 (Myf5), F (1, 43) = 10.42, p<0.05 (Figure 4.9A). Additionally, a drug effect was observed, with DOX-treated groups expressing higher Myf5, F (1, 43) = 4.267, p<0.05. No significant interaction was observed. *Post hoc* tests revealed that SED-SAL expressed significantly less Myf5 than EXER-DOX. In the EDL, no significant main effects were observed, and no interaction was detected (Figure 4.9B).

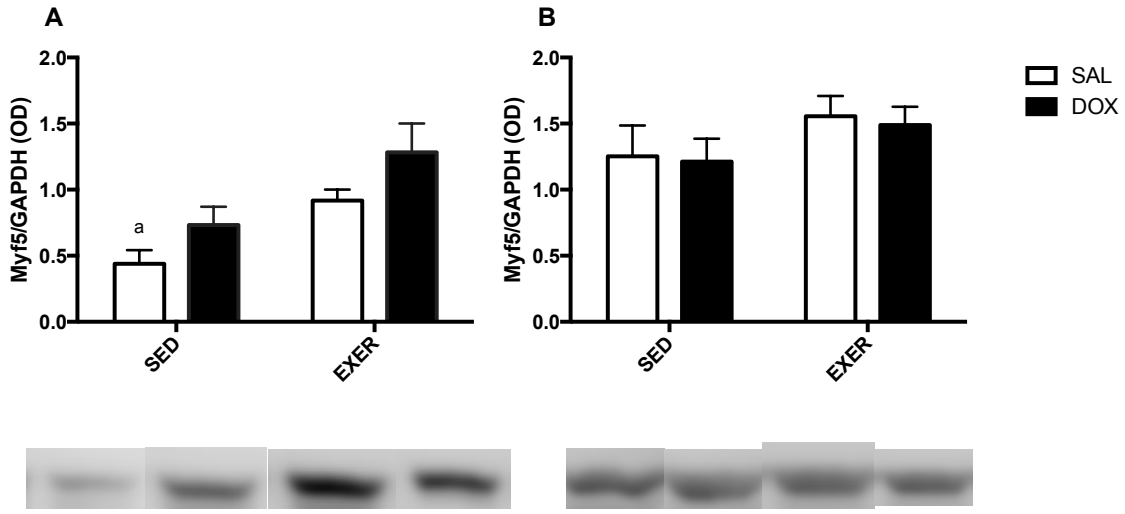

Figure 4.9. Myf5 expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-DOX.
Significant activity and drug effect in SOL Myf5 (p<0.05).

MyoD

In the SOL, a significant drug effect was observed, with DOX groups exhibiting higher levels of MyoD, F (1, 43) = 5.382, p<0.05 (Figure 4.10A). No activity effect and no interaction in SOL MyoD were detected. In the EDL, no significant main effects were identified, and no interaction was observed (Figure 4.10B).

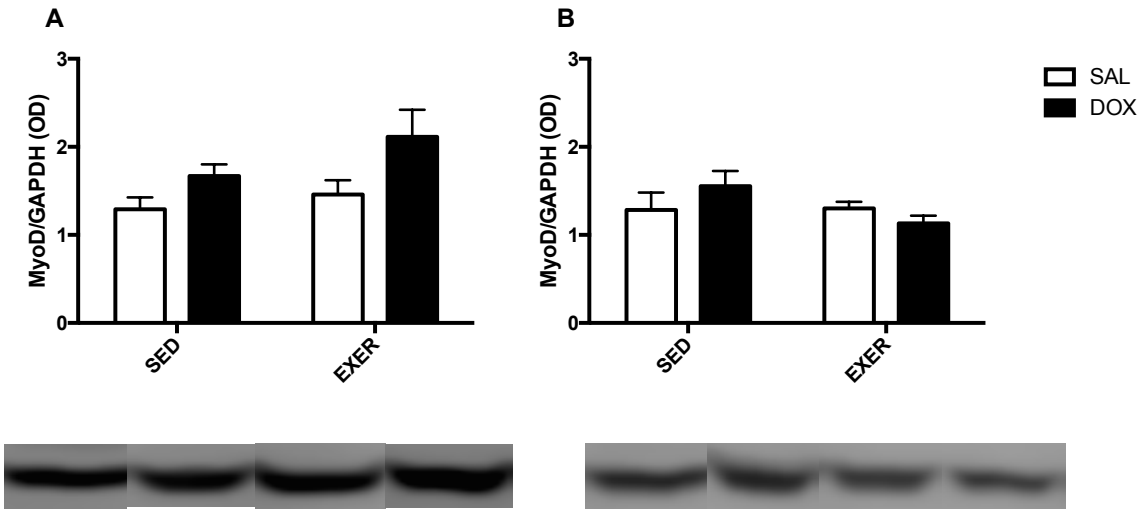

Figure 4.10. MyoD expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant drug effect in SOL MyoD (p<0.05).

Myogenin

In the SOL, no significant drug or activity effects or interaction were observed (Figure 4.11A). In the EDL, no significant main effects or interaction were identified (Figure 4.11B).

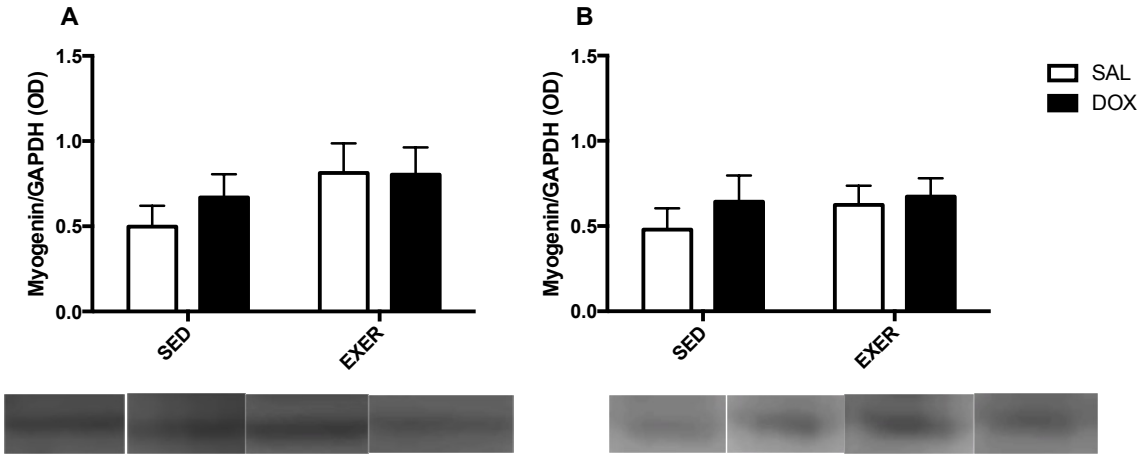

Figure 4.11. Myogenin expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Muscle Regulatory Factor 4

In the SOL, a significant activity effect was observed, with EXER groups exhibiting higher levels of muscle regulatory factor 4 (Mrf4), F (1, 43) = 5.672, $p<0.05$ (Figure 4.12A). No drug effect or interaction in the SOL was detected. In the EDL, no significant main effects were identified, and no interaction was observed either (Figure 4.12B).

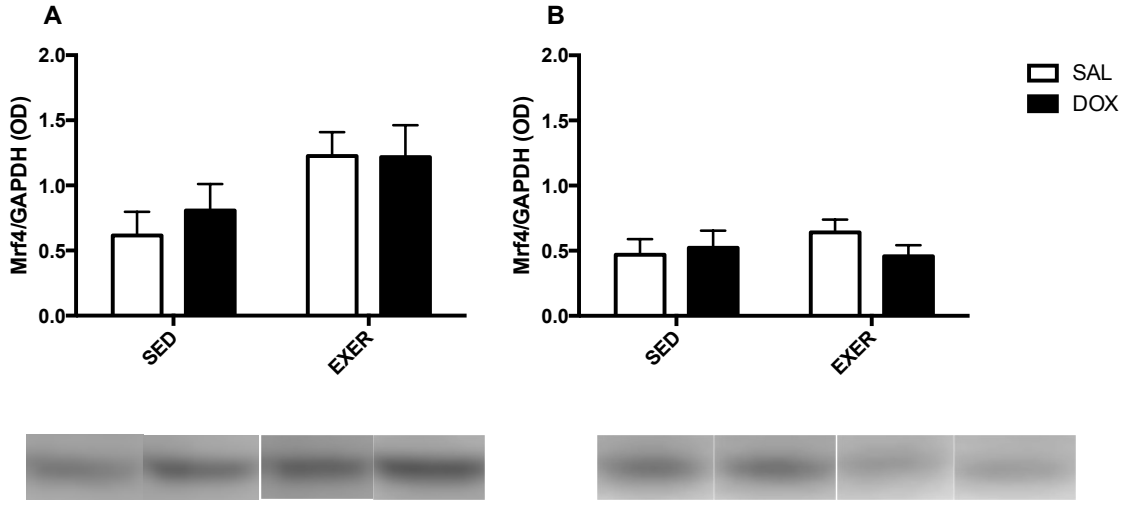

Figure 4.12. Mrf4 expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant activity effect in SOL Mrf4 ($p<0.05$)

Summary

Acute DOX treatment and short-term endurance exercise did not significantly affect skeletal muscle maximal twitch force production in the SOL or EDL. The time taken to fatigue in both SOL and EDL was longest in sedentary animals receiving saline injections. No absolute measures of tissue mass were significantly different but, after correcting for relative mass, an activity main effect was observed with higher EDL masses. No mass differences were seen in SOL.

An activity main effect in the EDL of EXER animals suggests elevated oxidative stress as indicated by higher levels of lipid peroxidation. Surprisingly, lipid peroxidation levels were higher in the SOL of SAL animals than SOL of DOX animals. Myf5 expression in the SOL was increased with both drug and exercise treatments. MyoD expression in the SOL was increased with DOX treatment. Levels of Mrf4 in the SOL

were elevated with endurance training. No change was seen in SOL myogenin levels, and no changes in Myf5, MyoD, Mrf4, or myogenin were observed in the EDL due to exercise or DOX treatments.

CHAPTER V

MANUSCRIPT FOR PUBLICATION

Abstract

Doxorubicin (DOX) is a widely used anthracycline antibiotic used to treat a number of hematological and solid tumor cancers. Dosage, however, is limited due to its toxic effects in healthy tissues. Negative consequences include myotoxicity in skeletal muscle, which may limit mobility and activities of daily living. The capacity for skeletal muscular regeneration relies heavily of the activity of myogenic regulatory factor (MRF) proteins. *In vitro* experiments with DOX depress the expression of MRFs but *in vivo* treatment may elicit different responses. Endurance exercise has been shown to elevate MRF expression, and may preserve MRFs following *in vivo* DOX-treatment.

Purpose: To determine the effect of short-term endurance training and acute DOX administration of skeletal muscle force production and fatigue resistance, levels of lipid peroxidation, and expression of MRFs. **Methods:** Ten week old male Sprague-Dawley rats were randomly assigned to one of four groups: sedentary + saline (SED-SAL), SED-DOX, endurance exercise training + saline (EXER-SAL), or EXER-DOX. Animals remained sedentary or performed treadmill training for two weeks. Twenty four hours after the activity period, animals were injected with a bolus i.p. injection of DOX (15 mg/kg) or SAL. Twenty four hours after injection, soleus (SOL) and extensor digitorum longus (EDL) skeletal muscles were removed for *ex vivo* function measures. Analyses of lipid peroxidation (malondialdehyde and 4-hydroxyalkenals [MDA + 4-HAE]) and

Western blotting for MRF expression (Myf5, MyoD, myogenin, Mrf4) were performed on contralateral muscles. Endurance exercise significantly elevated Myf5 and Mrf4 in the SOL ($p<0.05$). No significant differences existed in MRF expression levels in the EDL. No significant muscle force production or fatigue resistance differences were identified due to drug or activity treatment. MDA + 4-HAE was higher in the SOL of SAL animals ($p<0.05$) and EDL of EXER animals ($p<0.05$). **Conclusion:** Short-term endurance exercise elevated Myf5 and Mrf4 in slow, oxidative muscle after acute DOX treatment. Endurance exercise prior to chemotherapy may augment skeletal muscles' regenerative capacity following treatment, when loss of muscle mass is common.

Introduction

Muscle weakness and subsequent deterioration in activities of daily living are common side effects of chemotherapy treatments prescribed to cancer patients (Bonifati et al., 2000; Burckart, Beca, Urban, & Sheffield-Moore, 2010; Knobel et al., 2001). The commonly used anthracycline antibiotic, doxorubicin (DOX; trade name: Adriamycin®) has been shown to elicit consequences, which precede severe to fatal associations with its cardiotoxic nature. Free radicals formed by iron-catalyzed reactions are implicated in nuclear and mitochondrial damage inducing cell death (Bagchi, Bagchi, Hassoun, Kelly, & Stohs, 1995; DeAtley et al., 1999; Rapozzi et al., 1998; Stathopoulos et al., 1997). Much of the existing literature surrounding DOX-induced injury focuses on the effects seen in the heart. More recently, research has elucidated serious skeletal muscle harm, decreasing muscle size and function in response to DOX exposure (Doroshow, Tallent, & Schechter, 1985; Gilliam et al., 2009; Gilliam et al., 2013; Gilliam, Moylan, Callahan, Sumandea, & Reid, 2011). Previous research has demonstrated the beneficial influence of

endurance exercise preconditioning in mitigating the negative cardio- and myotoxic consequences of DOX treatment (Ascensão, Oliveira, & Magalhães, 2012; Chicco, Schneider, & Hayward, 2006; Hayward, Lien, Jensen, Hydock, & Schneider, 2012; Hydock, Lien, Jensen, Schneider, & Hayward, 2011b).

Skeletal muscle comprises a large part of the human body and is responsible for posture and locomotion. It is unique in its plasticity to alter its form following various stimuli. In adult muscle, myogenic regulatory factors (MRFs) guide satellite cells to restore muscle integrity in response to damage and stress, such as exercise. Activated satellite cells, expressing primary MRFs, form myoblasts, and differentiate into myotubes in response to secondary MRFs and reconstitute muscle fibers. The ability of skeletal muscle to repair itself and retain structure relies heavily on functional MRF proteins.

In vitro DOX exposure has been shown to decrease the ability of myoblasts to differentiate into myotubes (Kurabayashi, Jeyaseelan, & Kedes, 1993). Additionally, MRF mRNA expression is compromised under the same conditions with an up-regulation of the MRF inhibitor, Id (Kurabayashi, Jeyaseelan, & Kedes, 1994). DOX has been shown to induce oxidative stress, leading to cellular damage and single-stranded DNA breaks. Beyond oxidative damage, genotoxic stress attributed to DOX leads to double-stranded DNA breaks. The primary MRF, MyoD, is fundamentally involved in myoblast DNA repair (Kobayashi, Antoccia, Tauchi, Matsuura, & Komatsu, 2004). Its presence, along with the other MRFs, is critical for skeletal muscle regeneration following chemotherapy treatment which includes DOX. Prior endurance exercise training may also influence the expression of MRFs in skeletal muscle. Following endurance training, SOL of rats express elevated levels of oxidative enzyme genes and myogenin (Siu, Donley,

Bryner, & Alway, 2004). Although MRF mRNA has been broadly investigated, protein expression has been less examined.

The purpose of this study was to test whether a two-week treadmill protocol prior to acute DOX administration would preserve skeletal muscle function (twitch force and fatigue resistance) and decrease levels of lipid peroxidation. It was hypothesized that DOX treatment would impair skeletal muscle force production and fatigue resistantce while increasing lipid peroxidation. These decrements were predicted to be attenuated with short-term aerobic preconditioning. In combination with elevated antioxidant enzymes with short-term aerobic exercise, increased MRF protein may effectively mitigate the skeletal muscle dysfunction attributed to DOX treatment and enhance subsequent repairs. A tertiary hypothesis of this study postulated that exercise training would elevate MRF concentrations in the SOL and EDL.

Materials and Methods

Experimental Design

Ten week old male Sprague-Dawley rats (Harlan: Indianapolis, IN; N=47) were housed in pairs under a 12:12 hour light-dark cycle at room temperature (20 ± 2°C). Rats were provided food (Harlan Taklad 2026 rat chow) and distilled water *ad libitum*. All experimental procedures were approved by the University of Northern Colorado Institutional Animal Care and Use Committee (Protocol #1407C-DH-R-17) and were in compliance with the Animal Welfare Act guidelines.

Rats were randomly assigned to sedentary (SED) (n=20) or treadmill exercise (EXER) (n=27) groups. The SED group was limited to normal cage activity for the duration of the study. Animals in the EXER group were exercised progressively on a

motorized treadmill at 30 m/min through week 1 and for one hour during week 2 (Table 5.1). When necessary, rats were motivated by light electric shock at the rear of treadmill lanes. All EXER subjects completed the training protocol.

Table 5.1

Treadmill exercise training protocol

Day	1	2	3	4	5	6 – 7	8 - 12
Speed (m/min)	30	30	30	30	30	(Rest)	30
Duration (min)	10	20	30	40	50	(Rest)	60
Incline (%)	0	0	0	0	0	(Rest)	0

Drug Treatment

At the completion of the two week activity period, animals were sedentary for 24 hours. After the 24 hour sedentary period, animals were subdivided to receive DOX or saline (SAL) injections: SED-SAL (n=10), SED-DOX (n=10), EXER-SAL (n=13), EXER-DOX (n=14). Subjects in the DOX group received a bolus i.p. 15 mg/kg injection of DOX hydrochloride (Bedford Labs: Bedford, OH). Animals in the SAL group received an equivalent volume of 0.9% saline. 24 hours following injections, animals were sacrificed and muscles of interest were removed.

Tissue Preparation

Animals were anesthetized with sodium pentobarbital (50 mg/kg), and once a tail pinch reflex was absent, soleus (SOL) and extensor digitorum longus (EDL) muscles were excised. Muscles from the left leg were flash frozen in liquid nitrogen and stored at -80°C for later biochemical analysis. Muscles from the right leg were transferred to a

warm organ bath (Radnoti: Monrovia, CA) of Krebs Henseleit buffer (120 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 MgCl, 25 $NaHCO_3$, 17 glucose; in mM) for muscle function data collection.

Assessment of Skeletal Muscle Function

Functional muscle data were obtained via electrostimulation of tissues. Two electrodes surrounding the muscle in organ baths provided stimulation. Muscle contraction forces were recorded using PowerLab data acquisition hardware (ADInstruments: Colorado Springs, CO). Maximal twitch force was achieved by adjusting muscle for optimal length, and subsequent change in voltage applied (Grass Technologies: Warwick, RI).

The muscle stimulation methods follow the protocol reported by Hydock et al. (2011). Muscles were allowed to stabilize in a warmed (37°C) and oxygenated (95% O_2/5% CO_2) organ bath prior to functional experiments. Initial muscle tension was adjusted to 0.5g, and stimulated with a square-wave pulse duration of 0.5 ms at 40V. Muscle tension was increased by 0.2g per stimulation (2-minute rest) until twitch force reached a maximum. Next, optimal voltage was verified by increasing applied voltage by 5V, allowing the same 2-minute rest period. Once maximal twitch force was determined, the bath of Krebs-Henseleit buffer was cycled to ensure proper electrolyte balance for the ensuing fatigue recording. The muscle was allowed a recovery time of 30 minutes in fresh buffer before continuous stimulation cycling. Pulse duration was increased to 500 ms while maintaining the determined optimal length and voltage. The muscle was stimulated continuously every 1 second for 2 minutes to simulate fatiguing conditions (Hydock, Lien, Jensen, Schneider, & Hayward, 2011a). LabChart software

(ADInstruments) was used to analyze force data acquired.

Measures of maximal force, maximal rate of force development, and maximal rate of force decline were recorded during the single twitch force stimulation recordings. During the continuous, fatiguing protocol, force production was recorded in reference to baseline levels every 10 seconds, for a total of 100 seconds.

Biochemical Analysis

Flash frozen left hindlimb muscles were homogenized and sonicated in radioimmunoprecipitation assay (RIPA) buffer (10:1) and protease enzyme inhibitors (SigmaAldrich: St. Louis, MO). Homogenates were then spun in a microcentrifuge for 10 minutes at 3000*g* at 4°C. Total protein was quantified and standardized in samples according to the bicinchronic acid (BCA) assay (Smith et al., 1985).

Lipid Peroxidation

The most abundant product of LPO is malondialdehyde (MDA), and it is commonly assayed as an index of oxidative stress. LPO was determined using a commercially available assay kit (BioxyTech MDA-586, Oxis Research: Foster City, CA). Malondialdehyde and 4-hydroxyalkenals (MDA+4-HAE) were measured to indicate cellular lipid peroxidation. Two hundred µL of sample homogenate was added to a microcentrifuge tube. Six hundred-fifty µL of N-methyl-2-phenylindole in acetonitrile was added to the sample and briefly vortexed. One hundred-fifty µL of methanesulfonic acid was then be added and vortexed, followed by a 60 minute incubation period at 45°C. Following the incubation period, samples were centrifuged at 10,000*g* for 10 minutes to remove turbidity. Supernatants were then transferred to cuvettes for absorbency measurement using a spectrophotometer at 586 nm. Concentration of MDA+4-HAE was

estimated from a standard curve. Samples were run in duplicate and reassayed if samples varied by more than 5%.

Western Blotting

Western blot analysis was conducted on muscle samples for the presence of MRFs in the SOL and EDL. An equal volume of Lammeli buffer was added to samples, and samples were heated in boiling water for 2 minutes, then chilled on ice for 5 minutes before 46 μg of protein was loaded onto 4-20% gradient Tris-Glycine NuPage precast gels (LifeTechnologies: Carlsbad, CA). Gels were run at 125 constant voltage and 4 mA current for 2 hours in a Xcell II blot module (Invitrogen, LifeTechnologies). Proteins were transferred to 0.45 micron polyvinylidene fluoride (PVDF) membranes over 90 minutes at 25 volts and 100 mA. Band transfers to PVDF membranes were ensured by the presence of a SeeBlue® Plus2 protein ladder (Novex, LifeTechnologies). Membranes were blocked for 1 hour in 15 mL of TBST + 5% milk, and then incubated with gentle agitation overnight in 10 mL of primary antibodies. Membranes were then washed in TBST three times for 5 minutes, followed by incubation in appropriate species-specific secondary antibodies for 1 hour. After three more 5-minute washes in TBST, membranes were prepared for protein band detection.

Detection was executed by enhanced chemiluminescence (ECL) (C-Digit, Li-Cor: Lincoln, NE), and ImageJ software (NIH: Bethesda, MD) was used to quantify the protein bands. Immediately before chemiluminescent imaging, 1.5 mL of luminol and enhancer (SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate, ThermoScientific: Waltham, MA) was added to the membrane. The primary antibodies of interest included rabbit monoclonal MyoD, myogenin (Santa Cruz Biotechnology:

Dallas, TX), Myf5, and Mrf4 (Abcam: Cambridge, MA). The rabbit monoclonal anti-GAPDH (Abcam) was used as a loading control. Molecular weights of protein bands were ensured in reference to a MagicMark™ XP standard ladder (Novex, LifeTechnologies). Secondary antibodies (Santa Cruz Biotechnology) corresponded to associated species (rabbit) and include horseradish peroxidase (HRP) for adequate reactivity.

Statistical Analysis

Data were analyzed and presented using GraphPad Prism statistical software (GraphPad: LaJolla, CA). Variables analyzed were assessed as means ± standard error (mean±SE). A two-factor (Exercise X Drug) analysis of variance (ANOVA) was used to determine main effects and interactions of treatments in muscle mass, muscle force production, lipid peroxidation, and MRF concentration. If a significant F-value was observed, a Tukey *post-hoc* pair-wise comparison identified significant differences between groups. Variables included the four MRF proteins concentrations, muscle masses, and force parameters from *ex vivo* analysis in each of the groups (maximal twitch, maximal rate of force production, and maximal rate of force decline). Muscle fatigue responses were analyzed with maximal twitch force obtained every 10 seconds, and compared to baseline twitch force (0 sec). Fatigue was determined to be when force production fell below 75% of baseline values. For all procedures, significance was set at the $\alpha=0.05$ level.

Results

General Observations

Table 5.2 presents animal characteristics at the time of injection and at the time of sacrifice. At the time of injection, animal body mass was significantly different between groups. An activity effect was observed with SED animals exhibiting significantly greater body mass, F (1, 43) = 16.72, p<0.05. *Post hoc* testing revealed body mass to be higher in SED-SAL and SED-DOX than EXER-SAL, p<0.05.

Table 5.2

Animal Characteristics.

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Injection Mass (g)	359.5 ± 11.3 [a]	364.4 ± 9.9 [a]	322.1 ± 5.2	335.0 ± 6.8
Sacrifice Mass (g)	360.4 ± 11.9 [b]	353.9 ± 9.0 [b]	325.5 ± 6.3	328.5 ± 6.1
SOL Mass (mg)	134.9 ± 6.6	140.4 ± 4.4	131.5 ± 5.1	132.9 ± 4.6
EDL Mass (mg)	142.0 ± 4.8	138.3 ± 7.6	135.2 ± 3.6	140.9 ± 3.6

SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM.
[a] = Significantly greater than EXER-SAL (p<0.05).
[b] = Significantly greater than EXER-DOX (p<0.05).
Significant activity effect in injection and sacrifice body mass (p<0.05).

At the time of sacrifice, a similar activity effect existed with greater mass in SED animals, F (1, 43) = 13.42, p<0.05. *Post hoc* testing revealed that EXER-DOX body mass was significantly less than SED-SAL and SED-DOX, p<0.05. A significant drug effect was observed in body mass change as well, F (1.43) = 34.65, p<0.05 (Figure 5.1). Rats receiving SAL slightly gained body mass after injections (1%) while those receiving

DOX decreased body mass (2.5%). Within groups, body mass changes were not significantly different (p>0.05). Reductions in body mass of DOX-treated animals may be attributed to significantly less food intake within 24 hour following injections (Gilliam et al., 2009).

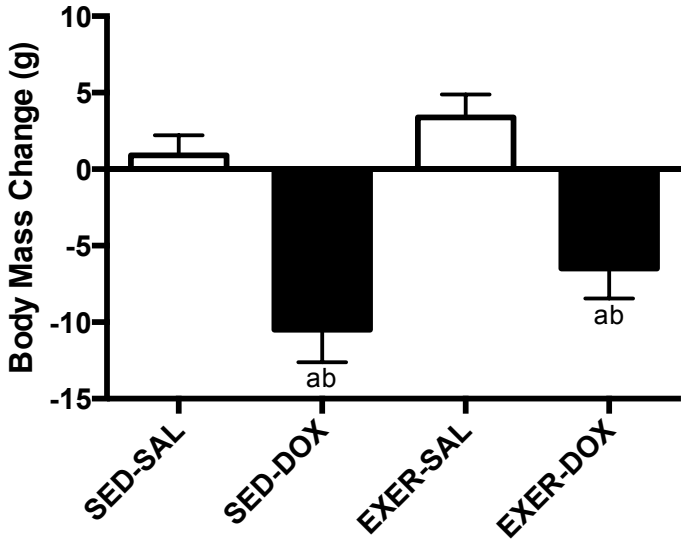

Figure 5.1. Change in body mass following injections.
SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL (p<0.05).
[b] = Significantly different from SED-SAL (p<0.05).

There was no significant difference in absolute SOL mass observed between groups, p>0.05 (Figure 5.2A). Similarly, when SOL mass was corrected for body mass, no significant difference was detected, p>0.05 (Table 5.3, Figure 5.3A). Absolute EDL masses displayed no significant differences for main effects or exercise by drug interaction, p<0.05 (Figure 5.2B). When corrected for body mass, an activity effect was

detected with endurance-trained animals demonstrating higher relative EDL mass, $F = (1, 43) = 4.871$, $p<0.05$ (Figure 5.3B).

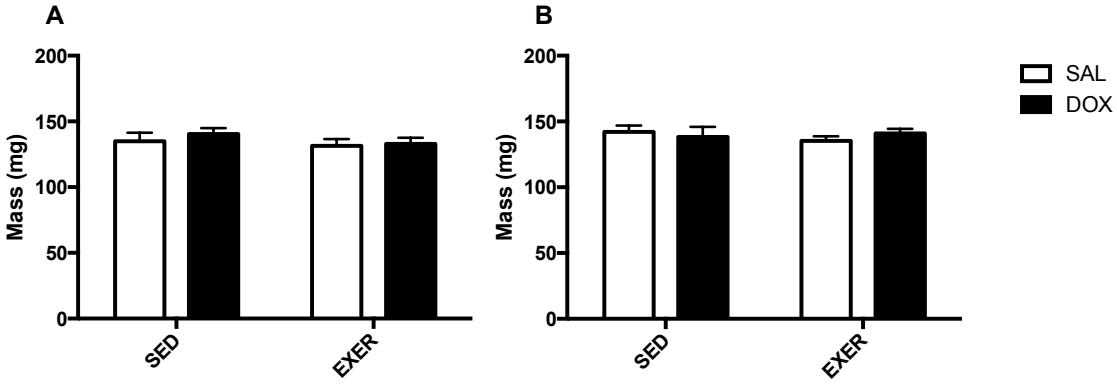

Figure 5.2. Tissue mass at time of sacrifice, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Table 5.3

Tissue mass relative to body mass.

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
SOL (mg/g BM)	0.37 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.41 ± 0.01
EDL (mg/g BM)	0.39 ± 0.01	0.39 ± 0.02	0.42 ± 0.01	0.43 ± 0.01

SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL ($p<0.05$).

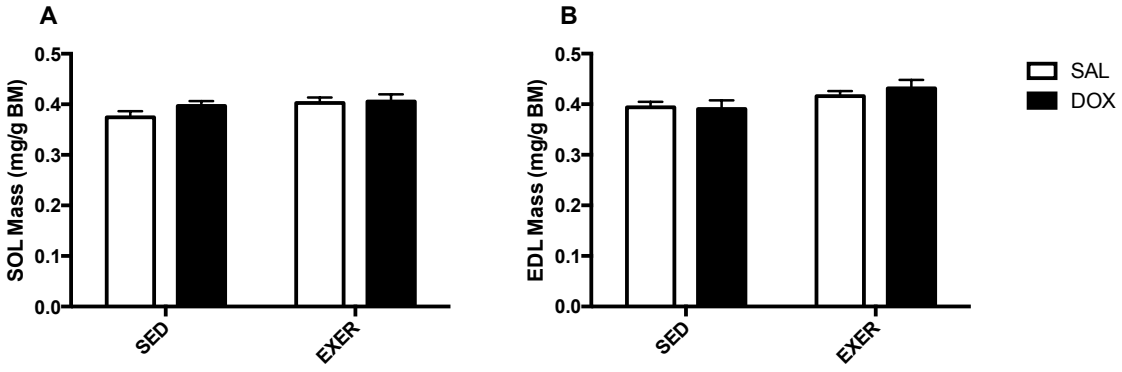

Figure 5.3. Tissue mass relative to body mass, SOL (A) and EDL (B). SOL = soleus, EDL = extensor digitorum longus, BM = body mass, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Isolated Muscle Function

Maximal Twitch Force. Once right SOL and EDL were excised, muscles were placed in organ baths filled with Krebs-Henseleit buffer. Tension and voltage were gradually increased to determine optimum conditions as detected by maximal twitch force elicited. Additionally, maximal rate of force production and maximal rate of force decline were measured from maximal twitch force tracings.

No significant differences were observed in SOL maximal twitch forces between groups, $p>0.05$ (Table 5.4). After adjusting force relative to tissue mass, significance remained undetected (Figure 5.4). Rates of force production and decline were also not significantly different between groups, $p<0.05$.

Table 5.4

SOL muscle twitch forces

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Maximal Twitch Force (mN)	37 ± 3	50 ± 5	44 ± 4	46 ± 4
Relative Maximal Twitch Force (mN/g)	249 ± 36	287 ± 29	258 ± 28	296 ± 41
Maximal Rate of Force Production (mN/s)	4916 ± 463	6376 ± 699	5478 ± 670	5536 ± 589
Maximal Rate of Force Decline (mN/s)	- 1715 ± 193	- 1935 ± 160	- 2027 ± 117	- 2255 ± 154

SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

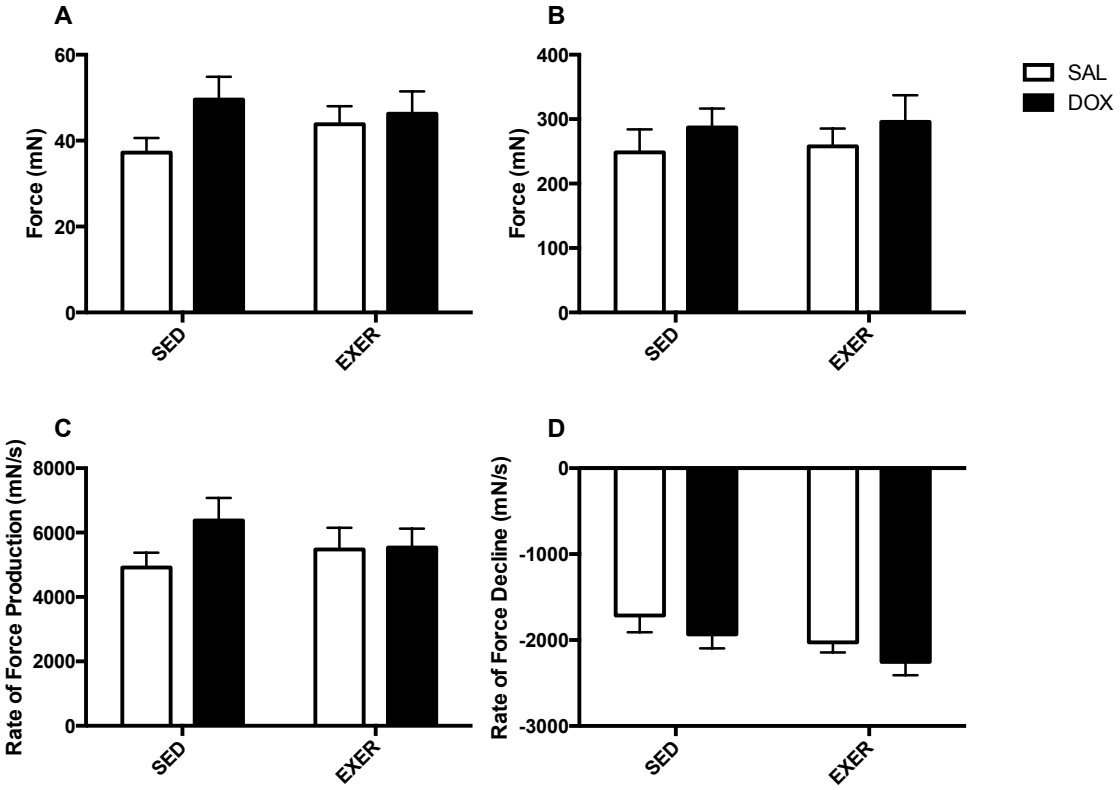

Figure 5.4. SOL Maximal twitch force values. Maximal twitch (A), maximal twitch relative to tissue mass (B), rate of force production (C), rate of force production (D), SOL rate of force decline.
SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

No significant differences were seen between groups in EDL maximal twitch forces, $p>0.05$ (Table 5.5). Similarly, no significance was observed after correcting for tissue mass. Rates of force production and decline were not significantly different between groups, as well (Figure 5.5).

Table 5.5

EDL muscle twitch forces

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Maximal Twitch Force (mN)	79 ± 7	90 ± 9	92 ± 8	77 ± 12
Relative Maximal Twitch Force (mN/g)	511 ± 26	603 ± 69	632 ± 51	545 ± 80
Maximal Rate of Force Production (mN/s)	14407 ± 1199	17851 ± 2060	18242 ± 1772	14966 ± 2603
Maximal Rate of Force Decline (mN/s)	- 8489 ± 690	- 10410 ± 1155	- 11583 ± 1272	- 9308 ± 1380

EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

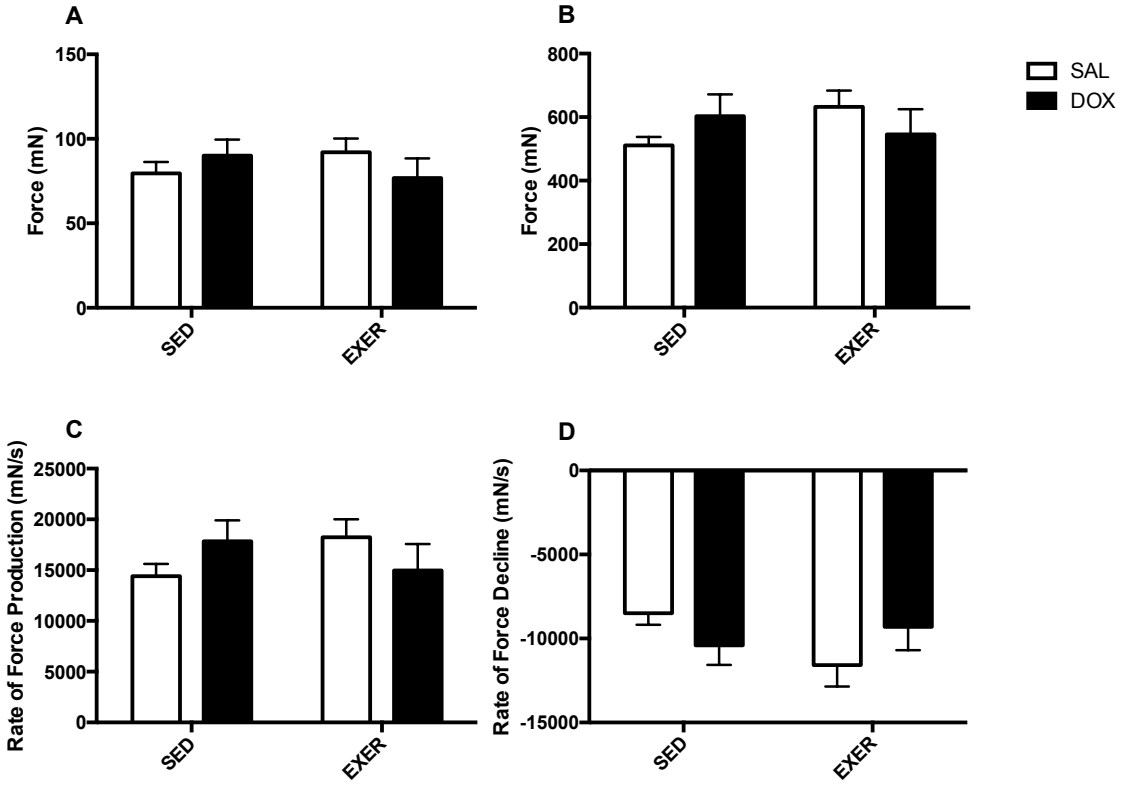

Figure 5.5. EDL Maximal twitch force values. Maximal twitch (A), maximal twitch relative to tissue mass (B), rate of force production (C), rate of force production (D), SOL rate of force decline.
EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Fatigue. After cycling Krebs-Henseleit buffer and allowing muscles to rest for 30 minutes, a 2-minute continuous stimulation at determined optimal length and voltage was performed. Time-to-fatigue was determined when muscles produced 75% of baseline force production.

In the SOL, SED-DOX and EXER-SAL generated less than 75% baseline force 40 seconds into fatiguing protocol. At the 50-second time point, EXER-DOX force production was below 75%. SED-SAL recorded the greatest fatigue resistance with twitch forces lower than 75% of baseline by 70 seconds (Figure 5.6).

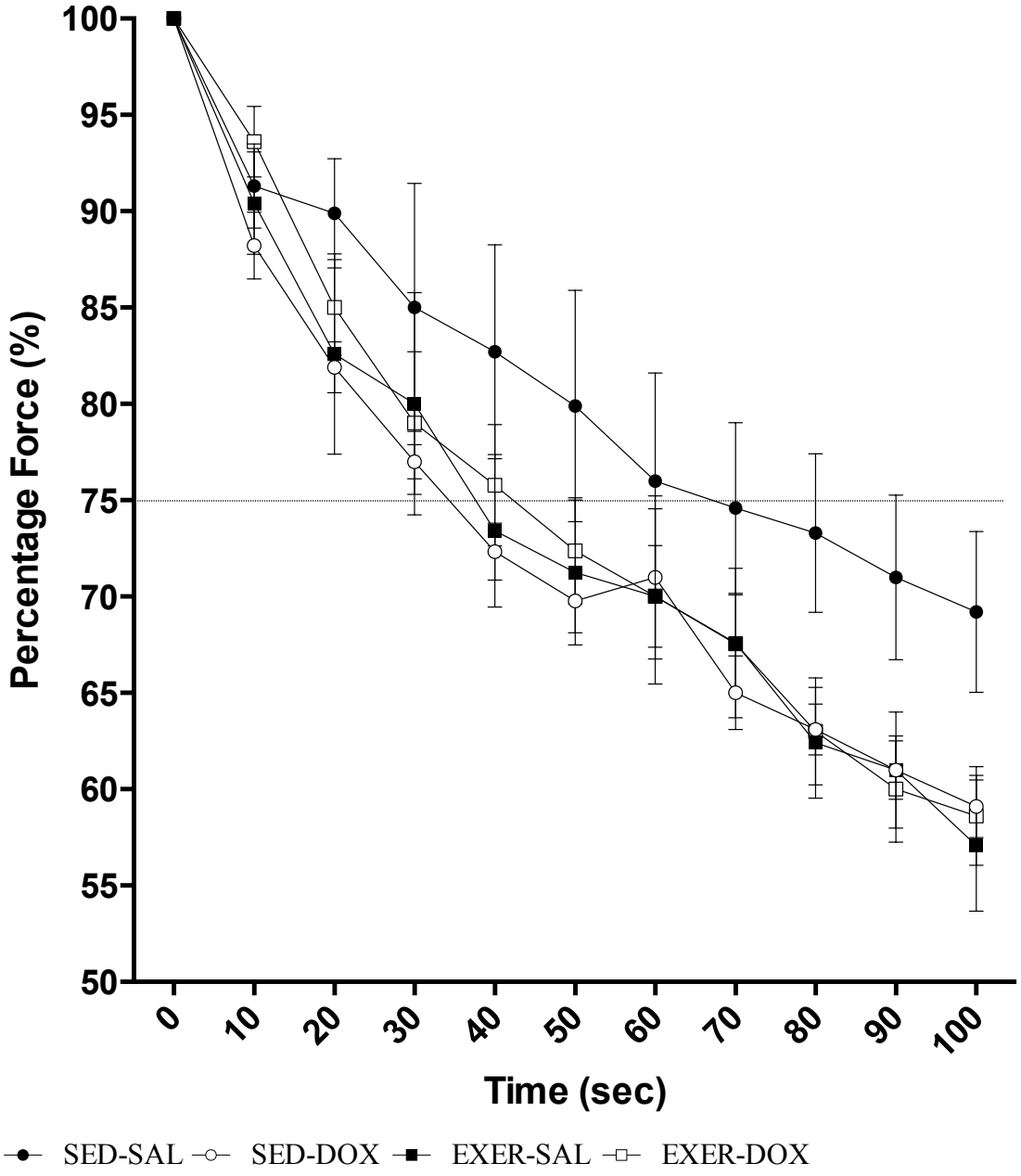

Figure 5.6. SOL fatigue resistance.
SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM.

In the EDL, SED-SAL fatigued quickest with twitch forces less than 75% at the 40-second time point. SED-DOX, EXER-SAL, and EXER-DOX groups' forces fell below fatigue threshold at 50 seconds (Figure 5.7). The fast-twitch muscle typically fatigues a greater rate than slow, oxidative muscles. *Ex vivo* force production of the EDL did not fatigue faster to levels below 75% of initial force values but, at the end 100 seconds, all groups' twitch forces were below those of the SOL.

The short-term duration of training may not have been long enough to accommodate appreciable fatigue resistance in SOL of EXER animals receiving DOX. Additionally, with greater time passing after DOX treatment functional deficits are evident. The 24 hours following injection may not have been enough to elucidate effects.

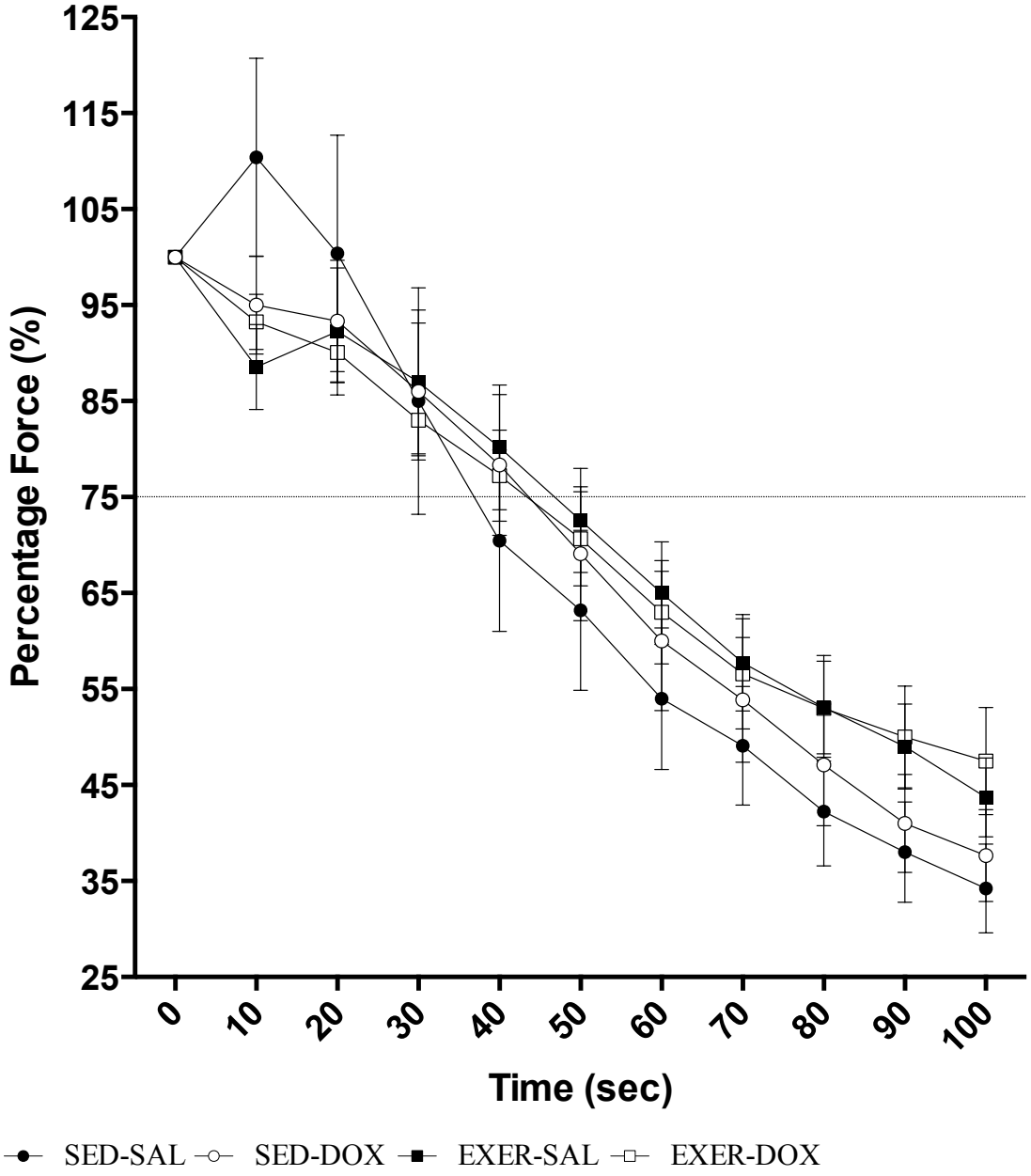

Figure 5.7. EDL fatigue resistance.
EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are means ± SEM.

Biochemical Analyses

Lipid Peroxidation. SOL and EDL homogenates obtained from rats 1 day after saline or DOX injection were analyzed for markers of lipid peroxidation (MDA+4-HAE). All data from LPO analysis are presented in Table 5.6. In SOL, a significant drug effect was observed. Rats receiving SAL exhibited higher levels of MDA+4-HAE than DOX animals, $F = (1, 43)$, $p<0.05$ (Figure 5.8A). *Post hoc* analyses identified EXER-SAL animals to express significantly greater indices of lipid peroxidation than SED-DOX and EXER-DOX. No activity effect or interaction was detected in the SOL. In the EDL, a significant activity effect was observed with TM animals presenting higher MDA+4-HAE, $F = (1, 43) = 4.08$, $p<0.05$ (Figure 5.8B). No drug effect or interaction was detected. The activity effect seen in EDL of EXER animals may have been due to stress of treadmill training on largely glycolytic muscles.

Table 5.6

Lipid peroxidation levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
SOL (pmol/mg)	329 ± 24	275 ± 17 [a]	398 ± 36	292 ± 17 [a]
EDL (pmol/mg)	214 ± 14	225 ± 17	283 ± 28	288 ± 44

SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL ($p<0.05$).
Significant drug effect in the SOL ($p<0.05$).
Significant activity effect in the EDL ($p<0.05$).

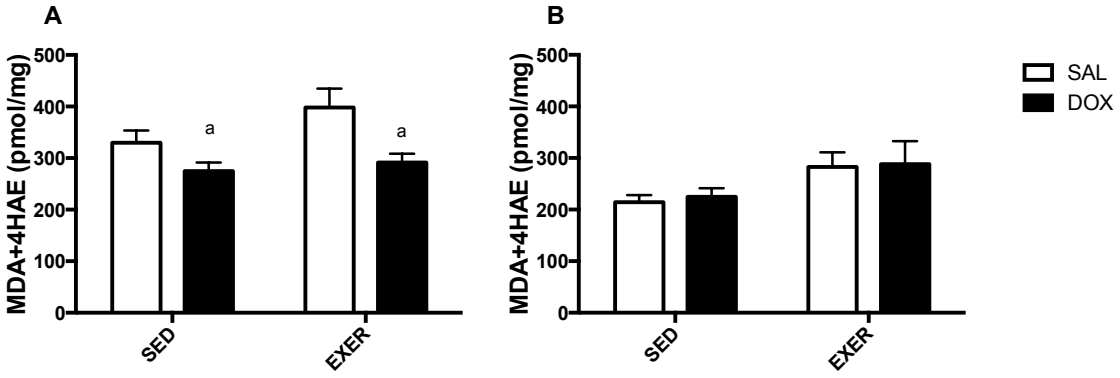

Figure 5.8. Lipid peroxidation levels in hindlimb muscles, SOL (A) and EDL (B). SOL = soleus, EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-SAL.
Significant drug effect in SOL ($p<0.05$).
Significant activity effect in EDL ($p<0.05$).

Western Blotting. Expression of myogenic regulatory factor proteins, Myf5, MyoD, Mrf4, and myogenin, were measured in SOL and EDL homogenates to evaluate the influence of endurance exercise and DOX on these transcription factors. Forty-six μg protein from SOL and EDL homogenates were added to 4-20% Tris-glycine precast gels and run through SDS-PAGE. MRF levels were assessed by chemiluminescence and expressed relative to GAPDH as a loading control. It should be noted that no significant GAPDH activity and drug main effects or interactions ($p>0.05$) were observed suggesting that the exercise or drug treatments did not affect the loading control. All Western blot data are presented in Tables 5.7 (SOL) and 5.8 (EDL).

Table 5.7

SOL MRF levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Myogenic Factor 5 (Myf5)	0.44 ± 0.10 [a]	0.73 ± 0.14	0.92 ± 0.08	1.28 ± 0.22
MyoD	1.29 ± 0.13	1.67 ± 0.13	1.46 ± 0.16	2.11 ± 0.31
Myogenin	0.50 ± 0.12	0.67 ± 0.14	0.81 ± 0.17	0.80 ± 0.16
Muscle Regulatory Factor 4 (Mrf4)	0.62 ± 0.18	0.80 ± 0.20	1.23 ± 0.18	1.22 ± 0.24

SOL = soleus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-DOX ($p<0.05$).
Significant activity and drug effect in Myf5 ($p<0.05$).
Significant drug effect in MyoD ($p<0.05$).
Significant effect effect in Mrf4 ($p<0.05$).

Table 5.8

EDL MRF levels

	SED-SAL	SED-DOX	EXER-SAL	EXER-DOX
Myogenic Factor 5 (Myf5)	1.25 ± 0.23	1.25 ± 0.19	1.57 ± 0.19	1.49 ± 0.14
MyoD	1.28 ± 0.20	1.55 ± 0.17	1.30 ± 0.07	1.13 ± 0.09
Myogenin	0.48 ± 0.15	0.64 ± 0.15	0.62 ± 0.11	0.67 ± 0.14
Muscle Regulatory Factor 4 (Mrf4)	0.47 ± 0.12	0.52 ± 0.13	0.64 ± 0.09	0.46 ± 0.08

EDL = extensor digitorum longus, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Myogenic Factor 5. In the SOL, a 2-way ANOVA revealed a significant activity effect, with EXER groups exhibiting higher levels of myogenic factor 5 (Myf5), F (1, 43) = 10.42, p<0.05 (Figure 5.9A). Additionally, a drug effect was observed, with DOX-treated groups expressing higher Myf5, F (1, 43) = 4.267, p=<0.05. No significant interaction was observed. *Post hoc* tests revealed that SED-SAL expressed significantly less Myf5 than EXER-DOX.

In the EDL, no significant main effects were observed (Figure 5.9B). No interaction was seen in the EDL, and no interaction was detected.

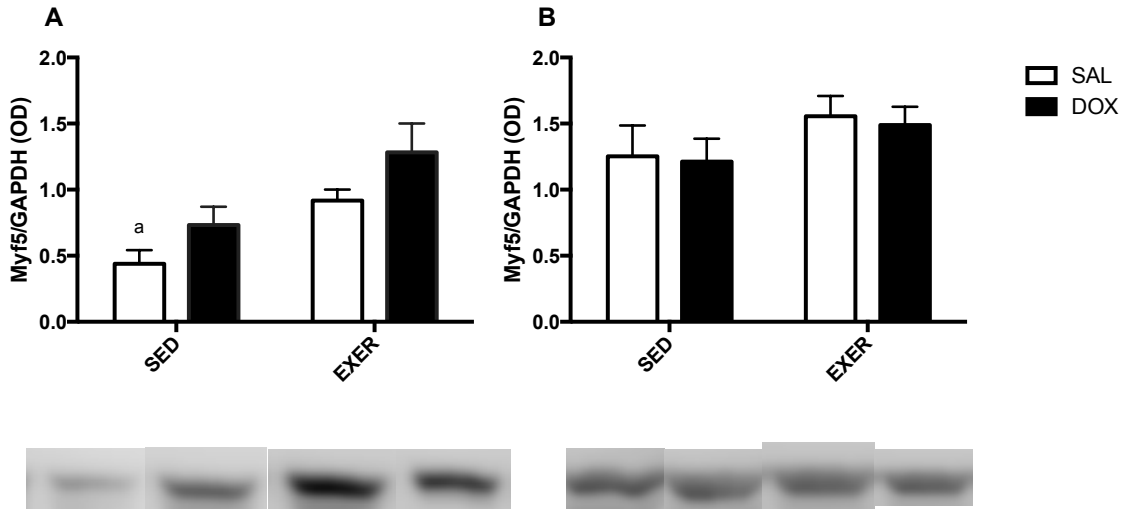

Figure 5.9. Myf5 expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
[a] = Significantly different from EXER-DOX.
Significant activity and drug effect in SOL Myf5 (p<0.05).

MyoD. In the SOL, a significant drug effect was observed, with DOX groups exhibiting higher levels of MyoD, F (1, 43) = 5.382, p<0.05 (Figure 5.10A). No activity effect and no interaction in SOL MyoD were detected. In the EDL, no significant main effects were identified. and no interaction was observed (Figure 5.10B).

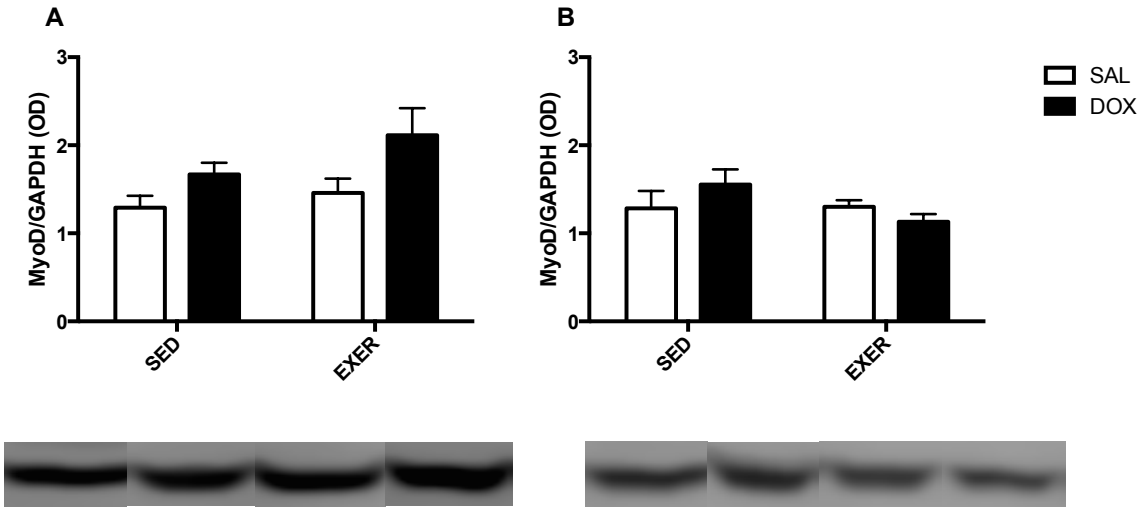

Figure 5.10. MyoD expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant drug effect in SOL MyoD (p<0.05).

Myogenin. In the SOL, no significant drug or activity effects were observed (Figure 5.11A). In the EDL, no significant main effects or interaction were identified (Figure 5.11B).

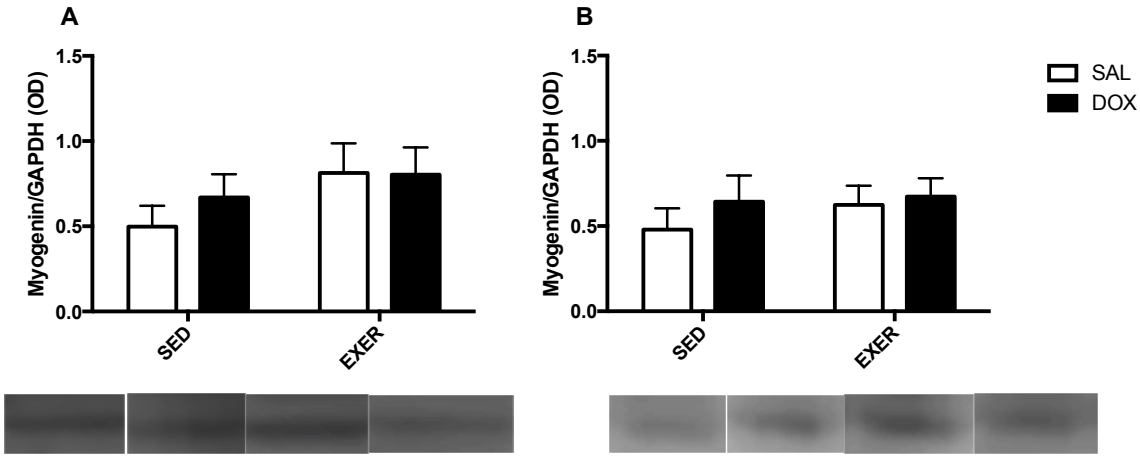

Figure 5.11. Myogenin expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.

Muscle Regulatory Factor 4. In the SOL, a significant activity effect was observed, with EXER groups exhibiting higher levels of muscle regulatory factor 4 (Mrf4), F (1, 43) = 5.672, p=0.0217 (Figure 5.12A). No drug effect or interaction in the SOL was detected. In the EDL, no significant main effects were identified, and no interaction was observed either (Figure 5.12B).

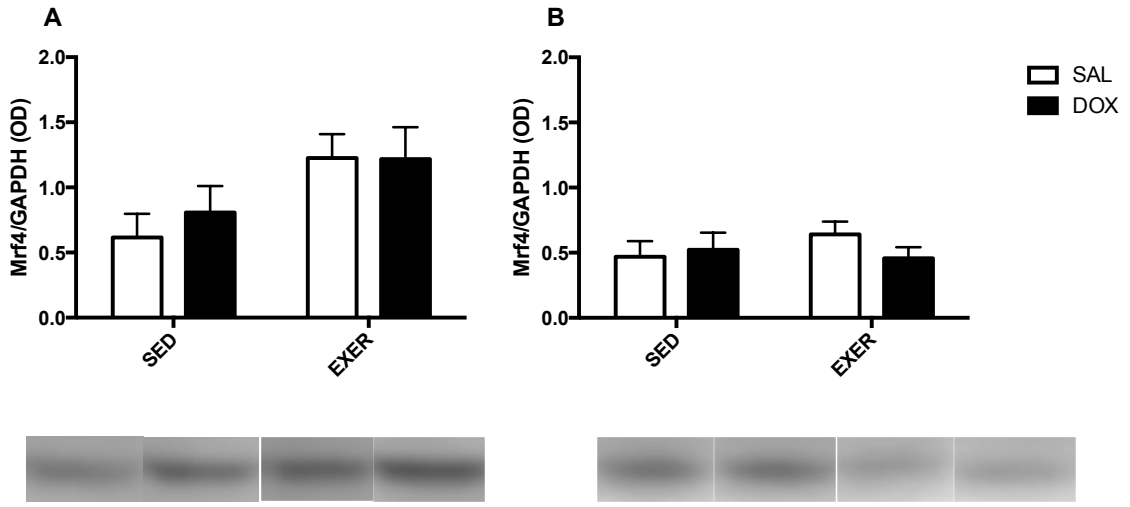

Figure 5.12. Mrf4 expression levels, SOL (A) and EDL (B).
SOL = soleus, EDL = extensor digitorum longus, OD = optical density, SED-SAL = sedentary saline, n=10; SED-DOX = sedentary doxorubicin, n=10; EXER-SAL = exercise saline, n=13; EXER-DOX = exercise doxorubicin, n=14. Values are mean ± SEM.
Significant activity effect in SOL Mrf4 ($p<0.05$).

Discussion

This is the first study to assess endurance exercise training and *in vivo* DOX effects on MRF expression in skeletal muscle. It was hypothesized that a two-week treadmill protocol prior to acute DOX administration would preserve muscle twitch forces and fatigue resistance, decrease levels of lipid peroxidation, and enhance MRF concentrations. The major findings are that 1) exercise did not alter muscle force parameters, 2) LPO was elevated in SAL-treated SOL and EXER group EDL, and 3) exercise training elevated Myf5 and Mrf4 levels in the SOL and acute DOX treatment increased Myf5 and MyoD in the SOL.

EXER animals had lower body masses than SED animals after two weeks of treadmill exercise, which may be attributed to increased caloric expenditure. Absolute and relative muscle tissue masses were not significantly reduced in DOX-treated groups

compared with acute SAL treatment. Previous studies identify DOX-induced decreases in muscle mass, but these losses are typically seen after 3 days (Doroshow et al., 1985). Within group body masses did not significantly differ, which is consistent with similar studies involving acute DOX exposure (Wonders, Hydock, Schneider, & Hayward, 2008).

Regarding skeletal muscle function, no significant differences were observed in either SOL or EDL as measures of *ex vivo* maximal twitch force. The present data suggest that acute DOX treatment did not reduce force production in hindlimb muscles one day following DOX injection. SED-DOX groups trended toward slightly better performance than SED-SAL in all maximal twitch measures (max force, rate of force development, rate of force decline). Previous studies have shown Ca^{2+} release to increase without changing Ca^{2+} sensitivity in muscle fibers exposed to DOX (Chugun et al., 2000; Zorzato, Salviati, Facchinetti, & Volpe, 1985). Failure of SR reuptake of Ca^{2+} may increase force produced during contractions, which may explain the trend toward slightly higher SED-DOX muscle twitch forces compared to SED-SAL (MacLennan, 2000).

Although two weeks of endurance exercise in SAL animals trended toward slightly higher force production, variables were not significantly different. DOX groups showed no significant differences in force production due to exercise preconditioning. In the SOL, SED-SAL took longest to fatigue below 75% of baseline force (70 sec), while SED-DOX and EXER-SAL fatigued quickest (40 sec). Conversely, the EDL of SED-SAL animals produced forces below the fatigue point (75%) ten seconds before all other groups (40 vs 50 sec). A study employing an equivalent dosage by Hydock et al. (2011) demonstrated significantly less twitch force, maximal rate of force production, and

maximal rate of force decline in DOX-treated animals five days following injections. The time period following DOX administration in this study may have been too brief to induce significant functional deficits. Additionally, exercise-induced injury of skeletal muscle without adequate time for recovery may explain EXER-SAL decrements in fatigue resistance of SOL. A previous study examining skeletal muscle damage associated with running reported degeneration, necrosis and phagocytosis shortly after and during 2 weeks following the onset of exercise (Irintchev & Wernig, 1987).

The generation of reactive oxygen species has been implicated as a mechanism of DOX's antineoplastic actions (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). As a side effect of DOX treatment, uncontrolled ROS production can induce LPO, damage to cell membranes and organelles, disrupted signaling pathways, apoptosis, and muscle atrophy (Chance, Sies, & Boveris, 1979). Additionally, ROS have been shown to stimulate Ca^{2+} release from skeletal muscle SR and affect the regulation of K^{+} channels in muscle tissues (Favero, Zable, & Abramson, 1995; Hool, Di Maria, Viola, & Arthur, 2005; Tang, Santarelli, Heinemann, & Hoshi, 2004). Interestingly, LPO levels in the SOL indicated a drug effect, with SAL groups presenting higher levels of MDA + 4-HAE. The predominantly fast, glycolytic EDL of EXER groups exhibited greater levels of lipid peroxidation than SED, which may be expected due to increased oxidative activity with treadmill running. ROS production signals pathways to promote skeletal muscle adaptations following exercise (Franco, Odom, & Rando, 1999; Li, Chen, Li, & Reid, 2003). If training periods were extended, a significant increase in SOL MDA+4-HAE content with exercise may have also been observed as a previous study by Liu et al. (2000) demonstrated both fast and slow muscle MDA content to be elevated following

chronic treadmill training in rats versus sedentary and acutely-trained animals. Human and rat studies suggest repeated exercise induces increased antioxidant levels and enzyme activity, which handle free radicals and reduce lipid peroxidation (Dekkers, Van Dooren, & Kemper, 1996). DOX administration did not elevate levels of MDA + 4-HAE at one day post-injection. The acute time point following injections (1 day) may not have allowed for significant DOX-associated lipid peroxidation of cell membranes to occur.

Expression of MRFs is required for the regeneration of muscle fibers in response to trauma or injury. These proteins direct satellite cells for myogenic lineage and differentiation into formed myotubes. In this study, MRFs were typically elevated in the SOL of DOX animals that performed endurance exercise compared to sedentary counterparts. Significant main activity effects were detected in Myf5 and Mrf4 with treadmill training. Myf5 may be critical to reestablish satellite cells for skeletal muscle regeneration following bouts of chemotherapy, and reducing muscle mass losses. The main activity effect was observed in DOX-treated rats that exercise trained as they expressed 75% more Myf5 than their SED counterparts. Additionally, EXER-SAL rats expressed 109% more Myf5 than SED-SAL.

A main activity effect was seen with increased Mrf4 expression following treadmill training. EXER-DOX expressed 51% more Mrf4 than SED-DOX. EXER-SAL rats expressed 99% more Mrf4 than SED-SAL. Increased Mrf4 expression may assist in terminal differentiation of new muscle cells. Additionally, Mrf4 has been shown to act upstream of MyoD determining cells for muscle identity (Kassar-Duchossoy et al., 2004). Taken together, exercise-induced elevations of these two MRFs (Myf5, Mrf4) posit the potential for greater muscle regeneration following DOX treatment versus sedentary

controls. Although not significant, EXER-DOX trended toward greater concentrations of MyoD and myogenin versus SED-DOX (27% and 20%, respectively).

Given the modality of endurance training, it is understandable that significant MRF alterations were observed in the slow, oxidative SOL versus the fast, glycolytic EDL. With running exercise, greater stress is placed on oxidative hindlimb muscles. Irintchev and Wernig (1987) indicated that voluntary running of mice induces damage in the SOL and tibialis anterior muscles, but not the EDL.

No main effects or interaction were observed in EDL. However, Myf5 and myogenin trended to slightly higher expression in EXER-DOX than SED-DOX groups (19% and 5%, respectively). In this study, overall MyoD levels were expressed at greater concentrations in the SOL than in the EDL (19%). Previous studies show higher MyoD mRNA levels in fast, glycolytic muscles (Hughes, Koishi, Rudnicki, & Maggs, 1997). The results of the present study may suggest that despite higher MyoD mRNA in Type II muscles, expression of the MyoD at the protein level may be higher in Type I fibers. Additionally, *in vitro* studies indicate a depression in MyoD when exposed to DOX (Kurabayashi et al., 1993, 1994). Conversely, our results present a drug effect with increased levels of MyoD expression in the SOL following DOX injections.

At time points greater than 24 hours following DOX treatment, reductions in MRF expression may occur as mass and function decrease but, the increased levels of Myf5 and Mrf4 may alleviate such losses. Along with CD34 and M-cadherin, Myf5 define quiescent satellite cells commited to myogenesis (Beauchamp et al., 2000). Decreased Myf5 expression in cultured cells is marked by decreased proliferation of satellite cell-derived myoblasts and delayed differentiation of myotubes (Ustanina,

Carvajal, Rigby, & Braun, 2007). Given the expression of Mrf4 at the time around and after fusion, elevated protein levels suggest increased skeletal muscle repair (Zhou & Bornemann, 2001). Endurance exercise-induced increases in Myf5 and Mrf4 potentially improve satellite cell populations and fusion of myotubes thereby attenuating muscle mass losses in the SOL after acute DOX treatment.

Conclusion

The present study examined the effects of short-term endurance exercise and acute DOX administration on skeletal muscle force production, fatigue resistance, LPO, and expression of MRFs. Although no remarkable findings were observed in muscle function or lipid peroxidation, a significant elevation in the expression of Myf5 and Mrf4 with endurance training in the SOL was revealed. Myf5 is suggested to replenish satellite cell pools and Mrf4 assists in the terminal differentiation of muscle cells (Rudnicki et al. 2008; Zhu & Miller, 1997). With increases in these two MRFs, the ability to regenerate and repair skeletal muscle may be enhanced in the time following chemotherapy, when loss in muscle mass is common. Future research examining parameters at later time points following treatment may elicit differential effects, but endurance exercise shows promise in upregulating some myogenic transcription factor expression (Myf5, Mrf4) in slow, oxidative muscles shortly after acute DOX treatment. Given the importance of time-to-chemotherapy and improved clinical outcomes, a short-term endurance exercise protocol prior to treatment may provide a feasible measure to prevent muscle mass losses seen with chemotherapy in cancer patients (de Melo Gagliato et al. 2014; Doroshow, et al., 1985; Gilliam et al., 2009; Gilliam et al., 2013; Gilliam et al., 2011).

REFERENCES

Anderson, E. J., & Neufer, P. D. (2006). Type II skeletal myofibers possess unique properties that potentiate mitochondrial H(2)O(2) generation. *American Journal of Physiology - Cell Physiology, 290*(3), C844-851. doi: 10.1152/ajpcell.00402.2005

Ascensão, A., Oliveira, P. J., & Magalhães, J. (2012). Exercise as a beneficial adjunct therapy during Doxorubicin treatment—Role of mitochondria in cardioprotection. *International Journal of Cardiology, 156*(1), 4-10.

Azmi, S., Ozog, A., & Taneja, R. (2004). Sharp-1/DEC2 inhibits skeletal muscle differentiation through repression of myogenic transcription factors. *Journal of Biological Chemistry, 279*(50), 52643-52652.

Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A., & Zammit, P. S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *Journal of Cell Biology, 151*(6), 1221-1234. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11121437 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2190588/pdf/0004041.pdf

Bagchi, D., Bagchi, M., Hassoun, E. A., Kelly, J., & Stohs, S. J. (1995). Adriamycin-induced hepatic and myocardial lipid peroxidation and DNA damage, and enhanced excretion of urinary lipid metabolites in rats. *Toxicology, 95*(1-3), 1-9.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., & Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell, 61*(1), 49-59. doi: http://dx.doi.org/10.1016/0092-8674(90)90214-Y

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor Perspectives in Biology, 4*(2), a008342. doi: 10.1101/cshperspect.a008342

Bickel, C. S., Slade, J., Mahoney, E., Haddad, F., Dudley, G. A., & Adams, G. R. (2005). Time course of molecular responses of human skeletal muscle to acute bouts of resistance exercise. *Journal of Applied Physiology (1985), 98*(2), 482-488. doi: 10.1152/japplphysiol.00895.2004

Biggs, J., Murphy, E. V., & Israel, M. A. (1992). A human Id-like helix-loop-helix protein expressed during early development. *Proceedings of the National Academy of Sciences of the United States of America, 89*(4), 1512-1516.

Bischoff, R. (1994). The satellite cell and muscle regeneration. *Myology, 1*, 97-118.

Black, B. L., & Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annual Review of Cell and Developmental Biology, 14*(1), 167-196. doi: 10.1146/annurev.cellbio.14.1.167

Bober, E., Lyons, G. E., Braun, T., Cossu, G., Buckingham, M., & Arnold, H. (1991). The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *Journal of Cell Biology, 113*(6), 1255-1265.

Bonifati, D. M., Ori, C., Rossi, C. R., Caira, S., Fanin, M., & Angelini, C. (2000). Neuromuscular damage after hyperthermic isolated limb perfusion in patients with melanoma or sarcoma treated with chemotherapeutic agents. *Cancer Chemotherapy and Pharmacology, 46*(6), 517-522. doi: 10.1007/s002800000175

Brand-Saberi, B., & Christ, B. (1999). Genetic and epigenetic control of muscle development in vertebrates. *Cell and Tissue Research, 296*(1), 199-212.

Braun, T., Rudnicki, M. A., Arnold, H.-H., & Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell, 71*(3), 369-382.

Buckingham, M. (2001). Skeletal muscle formation in vertebrates. *Current Opinion in Genetics and Development, 11*(4), 440-448.

Burckart, K., Beca, S., Urban, R. J., & Sheffield-Moore, M. (2010). Pathogenesis of muscle wasting in cancer cachexia: targeted anabolic and anticatabolic therapies. *Current Opinion in Clinical Nutrition and Metabolic Care, 13*(4), 410-416.

Chabner, B., Ryan, D., Paz-Ares, L., & Garcia-Carbonero, R. (2001). P. Calabresi in The Pharmacological Basis of Therapeutics: McGraw Hill, NY.

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews, 59*(3), 527-605.

Chandra, J., Samali, A., & Orrenius, S. (2000). Triggering and modulation of apoptosis by oxidative stress. *Free Radical Biology and Medicine, 29*(3-4), 323-333. doi: http://dx.doi.org/10.1016/S0891-5849(00)00302-6

Chi, N., & Epstein, J. A. (2002). Getting your Pax straight: Pax proteins in development and disease. *Trends in Genetics, 18*(1), 41-47.

Chicco, A. J., Hydock, D. S., Schneider, C. M., & Hayward, R. (2006). Low-intensity exercise training during doxorubicin treatment protects against cardiotoxicity. *Journal of Applied Physiology (1985), 100*(2), 519-527. doi: 10.1152/japplphysiol.00148.2005

Chicco, A. J., Schneider, C. M., & Hayward, R. (2006). Exercise training attenuates acute doxorubicin-induced cardiac dysfunction. *Journal of Cardiovascular Pharmacology, 47*(2), 182-189. doi: 10.1097/01.fjc.0000199682.43448.2d

Choi, J., Costa, M., Mermelstein, C., Chagas, C., Holtzer, S., & Holtzer, H. (1990). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proceedings of the National Academy of Sciences of the United States of America, 87*(20), 7988-7992.

Chu, E., & DeVita, V. T. (2006). *Physicians' cancer chemotherapy drug manual 2006*: Jones & Bartlett Learning.

Chugun, A., Temma, K., Oyamada, T., Suzuki, N., Kamiya, Y., Hara, Y., Sasaki, T., Kondo, H., & Akera, T. (2000). Doxorubicin-induced late cardiotoxicity: delayed impairment of Ca2+-handling mechanisms in the sarcoplasmic reticulum in the rat. *Canadian Journal of Physiology and Pharmacology, 78*(4), 329-338.

Ciccia, A., & Elledge, S. J. (2010). The DNA damage response: making it safe to play with knives. *Molecular Cell, 40*(2), 179-204. doi: 10.1016/j.molcel.2010.09.019

Corbi, N., Di Padova, M., De Angelis, R., Bruno, T., Libri, V., Iezzi, S., Floridi, A., Fanciulli, M., & Passananti, C. (2002). The α-like RNA polymerase II core subunit 3 (RPB3) is involved in tissue-specific transcription and muscle differentiation via interaction with the myogenic factor Myogenin. *The Federation of American Societies for Experimental Biology Journal, 16*(12), 1639-1641.

Cornelison, D. D., & Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Developmental Biology, 191*(2), 270-283. doi: 10.1006/dbio.1997.8721

Cui, H., Schroering, A., & Ding, H.-F. (2002). p53 Mediates DNA Damaging Drug-induced Apoptosis through a Caspase-9-dependent Pathway in SH-SY5Y Neuroblastoma Cells 1 Supported in part by a grant from the Ohio Cancer Research Associates and Howard Temin Award CA-78534 from the National Cancer Institute, NIH. 1. *Molecular Cancer Therapeutics, 1*(9), 679-686.

Davies, K. J., & Doroshow, J. H. (1986). Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. *Journal of Biological Chemistry, 261*(7), 3060-3067.

Davis, R. L., Cheng, P. F., Lassar, A. B., & Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell, 60*(5), 733-746.

de Melo Gagliato, D., Gonzalez-Angulo, A. M., Lei, X., Theriault, R. L., Giordano, S. H., Valero, V., Hortobagyi, G. N., & Chavez-MacGregor, M. (2014). Clinical impact of delaying initiation of adjuvant chemotherapy in patients with breast cancer. *Journal of Clinical Oncology*, JCO. 2013.2049. 7693.

DeAtley, S. M., Aksenov, M. Y., Aksenova, M. V., Harris, B., Hadley, R., Cole Harper, P., Carney, J. M., & Butterfield, D. A. (1999). Antioxidants protect against reactive oxygen species associated with adriamycin-treated cardiomyocytes. *Cancer Letters, 136*(1), 41-46.

Dedkov, E. I., Kostrominova, T. Y., Borisov, A. B., & Carlson, B. M. (2001). Reparative myogenesis in long-term denervated skeletal muscles of adult rats results in a reduction of the satellite cell population. *The Anatomical Record, 263*(2), 139-154.

Dedkov, E. I., Kostrominova, T. Y., Borisov, A. B., & Carlson, B. M. (2003). MyoD and myogenin protein expression in skeletal muscles of senile rats. *Cell and Tissue Research, 311*(3), 401-416.

Dekkers, J. C., van Doornen, L. J., & Kemper, H. C. (1996). The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports Medicine, 21*(3), 213-238.

Dietrich, S., Schubert, F. R., Gruss, P., & Lumsden, A. (1999). The role of the notochord for epaxial myotome formation in the mouse. *Cellular and Molecular Biology (Noisy-le-grand), 45*(5), 601-616.

Dimeo, F., Fetscher, S., Lange, W., Mertelsmann, R., & Keul, J. (1997). Effects of aerobic exercise on the physical performance and incidence of treatment-related complications after high-dose chemotherapy. *Blood*, *90*(9), 3390-3394.

Dimeo,F., Rumberger, B.G., & Keul, J. (1998). Aerobic exercise as therapy for cancer fatigue. *Medicine and Science in Sports and Exercise*, *30*, 475-478.

Doroshow, J. H., & Davies, K. J. (1986). Redox cycling of anthracyclines by cardiac mitochondria. II. Formation of superoxide anion, hydrogen peroxide, and hydroxyl radical. *Journal of Biolical Chemistry, 261*(7), 3068-3074.

Doroshow, J. H., Tallent, C., & Schechter, J. E. (1985). Ultrastructural features of Adriamycin-induced skeletal and cardiac muscle toxicity. *American Journal of Pathology, 118*(2), 288-297.

Edmondson, D. G., Cheng, T. C., Cserjesi, P., Chakraborty, T., & Olson, E. N. (1992). Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. *Molecular and Cellular Biology, 12*(9), 3665-3677.

Evans, W. J. (2004). Protein nutrition, exercise and aging. *Journal of the American College of Nutrition, 23*(6 Suppl), 601S-609S.

Favero, T. G., Zable, A. C., & Abramson, J. J. (1995). Hydrogen peroxide stimulates the Ca2+ release channel from skeletal muscle sarcoplasmic reticulum. *Journal of Biological Chemistry, 270*(43), 25557-25563.

Feng Gao, C., Ren, S., Zhang, L., Nakajima, T., Ichinose, S., Hara, T., Koike, K., & Tsuchida, N. (2001). Caspase-Dependent Cytosolic Release of Cytochrome c and Membrane Translocation of Bax in p53-Induced Apoptosis. *Experimental Cell Research, 265*(1), 145-151.

Fortini, P., Ferretti, C., Pascucci, B., Narciso, L., Pajalunga, D., Puggioni, E. M., Castino, R., Isidoro, C., Crescenzi, M., & Dogliotti, E. (2012). DNA damage response by single-strand breaks in terminally differentiated muscle cells and the control of muscle integrity. *Cell Death and Differentiation, 19*(11), 1741-1749. doi: 10.1038/cdd.2012.53

Franco, A. A., Odom, R. S., & Rando, T. A. (1999). Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. *Free Radical Biology and Medicine, 27*(9-10), 1122-1132.

Fulda, S., & Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene, 25*(34), 4798-4811. doi: 10.1038/sj.onc.1209608

Gewirtz, D. A. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical Pharmacology, 57*(7), 727-741.

Gilliam, L. A., Ferreira, L. F., Bruton, J. D., Moylan, J. S., Westerblad, H., St Clair, D. K., & Reid, M. B. (2009). Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. *Journal of Applied Physiology (1985), 107*(6), 1935-1942. doi: 10.1152/japplphysiol.00776.2009

Gilliam, L. A., Fisher-Wellman, K. H., Lin, C. T., Maples, J. M., Cathey, B. L., & Neufer, P. D. (2013). The anticancer agent doxorubicin disrupts mitochondrial energy metabolism and redox balance in skeletal muscle. *Free Radical Biology and Medicine, 65*, 988-996. doi: 10.1016/j.freeradbiomed.2013.08.191

Gilliam, L. A., Moylan, J. S., Ann Callahan, L., Sumandea, M. P., & Reid, M. B. (2011). Doxorubicin causes diaphragm weakness in murine models of cancer chemotherapy. *Muscle and Nerve, 43*(1), 94-102.

Gilliam, L. A., Moylan, J. S., Patterson, E. W., Smith, J. D., Wilson, A. S., Rabbani, Z., & Reid, M. B. (2012). Doxorubicin acts via mitochondrial ROS to stimulate catabolism in C2C12 myotubes. *American Journal of Physiology - Cell Physiology, 302*(1), C195-202. doi: 10.1152/ajpcell.00217.2011

Gomez-Cabrera, M., Domenech, E., Ji, L., & Viña, J. (2006). Exercise as an antioxidant: it up-regulates important enzymes for cell adaptations to exercise. *Science and Sports, 21*(2), 85-89.

Gomez-Cabrera, M.-C., Domenech, E., & Viña, J. (2008). Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radical Biology and Medicine, 44*(2), 126-131.

Gossett, L. A., Kelvin, D. J., Sternberg, E., & Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Molecular and Cellular Biology, 9*(11), 5022-5033.

Gutierrez, P. L. (2000). The metabolism of quinone-containing alkylating agents: free radical production and measurement. *Frontiers in Bioscience, 5*, 629-638.

Haddad, F., & Adams, G. R. (2002). Selected contribution: acute cellular and molecular responses to resistance exercise. *Journal of Applied Physiology (1985), 93*(1), 394-403. doi: 10.1152/japplphysiol.01153.2001

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., & Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science, 267*(5200), 1018-1021.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., & Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature, 364*(6437), 501-506. doi: 10.1038/364501a0

Hauschka, S. (1994). The embryonic origin of muscle. *Myology, 1*, 3-73.

Hayward, R., Hydock, D., Gibson, N., Greufe, S., Bredahl, E., & Parry, T. (2013). Tissue retention of doxorubicin and its effects on cardiac, smooth, and skeletal muscle function. *Journal of Physiology and Biochemistry, 69*(2), 177-187. doi: 10.1007/s13105-012-0200-0

Hayward, R., Lien, C. Y., Jensen, B. T., Hydock, D. S., & Schneider, C. M. (2012). Exercise training mitigates anthracycline-induced chronic cardiotoxicity in a juvenile rat model. *Pediatric Blood and Cancer, 59*(1), 149-154. doi: 10.1002/pbc.23392

Hidalgo, C., Bull, R., Behrens, M. I., & Donoso, P. (2004). Redox regulation of RyR-mediated Ca2+ release in muscle and neurons. *Biological Research, 37*(4), 539-552.

Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., & Konieczny, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Developmental Biology, 147*(1), 144-156.

Hool, L. C., Di Maria, C. A., Viola, H. M., & Arthur, P. G. (2005). Role of NAD(P)H oxidase in the regulation of cardiac L-type Ca2+ channel function during acute hypoxia. *Cardiovascular Research, 67*(4), 624-635. doi: 10.1016/j.cardiores.2005.04.025

Hoshi, T., & Lahiri, S. (2004). Cell biology. Oxygen sensing: it's a gas! *Science, 306*(5704), 2050-2051. doi: 10.1126/science.1107069

Hughes, S. M., Chi, M. M., Lowry, O. H., & Gundersen, K. (1999). Myogenin induces a shift of enzyme activity from glycolytic to oxidative metabolism in muscles of transgenic mice. *Journal of Cell Biology, 145*(3), 633-642.

Hughes, S. M., Koishi, K., Rudnicki, M., & Maggs, A. M. (1997). MyoD protein is differentially accumulated in fast and slow skeletal muscle fibres and required for normal fibre type balance in rodents. *Mechanisms of Development, 61*(1), 151-163.

Hughes, S. M., Taylor, J. M., Tapscott, S. J., Gurley, C. M., Carter, W. J., & Peterson, C. A. (1993). Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development, 118*(4), 1137-1147.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Schneider, C. M., & Hayward, R. (2011a). Characterization of the effect of in vivo doxorubicin treatment on skeletal muscle function in the rat. *Anticancer Research, 31*(6), 2023-2028.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Schneider, C. M., & Hayward, R. (2011b). Exercise preconditioning provides long-term protection against early chronic doxorubicin cardiotoxicity. *Integrative Cancer Therapies, 10*(1), 47-57. doi: 10.1177/1534735410392577

Ishibashi, J., Perry, R. L., Asakura, A., & Rudnicki, M. A. (2005). MyoD induces myogenic differentiation through cooperation of its NH2-and COOH-terminal regions. *Journal of Cell Biology, 171*(3), 471-482.

Ishido, M., Kami, K., & Masuhara, M. (2004). Localization of MyoD, myogenin and cell cycle regulatory factors in hypertrophying rat skeletal muscles. *Acta Physiologica Scandinavica, 180*(3), 281-289. doi: 10.1046/j.0001-6772.2003.01238.x

Irintchev, A., & Wernig, A. (1987). Muscle damage and repair in voluntarily running mice: strain and muscle differences. *Cell and Tissue Research, 249*(3), 509-521.

Ji, L. L., Gomez-Cabrera, M. C., & Vina, J. (2006). Exercise and hormesis: activation of cellular antioxidant signaling pathway. *Annals of the New York Academy of Sciences, 1067*(1), 425-435. doi: 10.1196/annals.1354.061

Kanter, M. M., Hamlin, R. L., Unverferth, D. V., Davis, H. W., & Merola, A. J. (1985). Effect of exercise training on antioxidant enzymes and cardiotoxicity of doxorubicin. *Journal of Applied Physiology (1985), 59*(4), 1298-1303.

Karalaki, M., Fili, S., Philippou, A., & Koutsilieris, M. (2009). Muscle regeneration: cellular and molecular events. *In Vivo, 23*(5), 779-796.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., & Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature, 431*(7007), 466-471. doi: 10.1038/nature02876

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Research, 51*(23 Pt 1), 6304-6311.

Kavazis, A. N., Smuder, A. J., & Powers, S. K. (2014). Effects of short-term endurance exercise training on acute doxorubicin-induced FoxO transcription in cardiac and skeletal muscle. *Journal of Applied Physiology (1985), 117*(3), 223-230. doi: 10.1152/japplphysiol.00210.2014

Knobel, H., Loge, J. H., Lund, M. B., Forfang, K., Nome, O., & Kaasa, S. (2001). Late medical complications and fatigue in Hodgkin's disease survivors. *Journal of Clinical Oncology, 19*(13), 3226-3233.

Kobayashi, J., Antoccia, A., Tauchi, H., Matsuura, S., & Komatsu, K. (2004). NBS1 and its functional role in the DNA damage response. *DNA Repair (Amst), 3*(8-9), 855-861. doi: 10.1016/j.dnarep.2004.03.023

Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K.-i., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K., & Komatsu, K. (2002). NBS1 Localizes

to γ-H2AX Foci through Interaction with the FHA/BRCT Domain. *Current Biology, 12*(21), 1846-1851. doi: 10.1016/s0960-9822(02)01259-9

Kosek, D. J., Kim, J. S., Petrella, J. K., Cross, J. M., & Bamman, M. M. (2006). Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *Journal of Applied Physiology (1985), 101*(2), 531-544. doi: 10.1152/japplphysiol.01474.2005

Kurabayashi, M., Jeyaseelan, R., & Kedes, L. (1993). Antineoplastic agent doxorubicin inhibits myogenic differentiation of C2 myoblasts. *Journal of Biological Chemistry, 268*(8), 5524-5529.

Kurabayashi, M., Jeyaseelan, R., & Kedes, L. (1994). Doxorubicin represses the function of the myogenic helix-loop-helix transcription factor MyoD. Involvement of Id gene induction. *Journal of Biological Chemistry, 269*(8), 6031-6039.

L'Ecuyer, T., Sanjeev, S., Thomas, R., Novak, R., Das, L., Campbell, W., & Vander Heide, R. (2006). DNA damage is an early event in doxorubicin-induced cardiac myocyte death. *American Journal of Physiology - Heart and Circulatory Physiology, 291*(3), H1273-H1280.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., & Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell, 66*(2), 305-315. doi: http://dx.doi.org/10.1016/0092-8674(91)90620-E

Lawler, J. M., Powers, S. K., Hammeren, J., & Martin, A. D. (1993). Oxygen cost of treadmill running in 24-month-old Fischer-344 rats. *Medicine and Science in Sports and Exercise, 25*(11), 1259-1264.

Lemercier, C., To, R. Q., Carrasco, R. A., & Konieczny, S. F. (1998). The basic helix–loop–helix transcription factor Mist1 functions as a transcriptional repressor of MyoD. *The European Molecular Biology Organization Journal, 17*(5), 1412-1422.

Li, Y. P., Chen, Y., Li, A. S., & Reid, M. B. (2003). Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *American Journal of Physiology - Cell Physiology, 285*(4), C806-812. doi: 10.1152/ajpcell.00129.2003

Lin, Q., Schwarz, J., Bucana, C., & Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science, 276*(5317), 1404-1407.

Liu, J., Yeo, H. C., Övervik-Douki, E., Hagen, T., Doniger, S. J., Chu, D. W., Brooks, G. A., & Ames, B. N. (2000). Chronically and acutely exercised rats: biomarkers of

oxidative stress and endogenous antioxidants. *Journal of Applied Physiology, 89*(1), 21-28. Retrieved from http://jap.physiology.org/content/jap/89/1/21.full.pdf

Loughna, P. T., & Brownson, C. (1996). Two myogenic regulatory factor transcripts exhibit muscle-specific responses to disuse and passive stretch in adult rats. *Federation of European Biochemical Societies Letters, 390*(3), 304-306.

Lowe, D. A., & Alway, S. E. (1999). Stretch-induced myogenin, MyoD, and MRF4 expression and acute hypertrophy in quail slow-tonic muscle are not dependent upon satellite cell proliferation. *Cell and Tissue Research, 296*(3), 531-539.

Lu, J., Webb, R., Richardson, J. A., & Olson, E. N. (1999). MyoR: A muscle-restricted basic helix–loop–helix transcription factor that antagonizes the actions of MyoD. *Proceedings of the National Academy of Sciences of the United States of America, 96*(2), 552-557.

Lyons, G. E., Micales, B. K., Schwarz, J., Martin, J. F., & Olson, E. N. (1995). Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. *Journal of Neuroscience, 15*(8), 5727-5738.

MacLennan, D. H. (2000). Ca2+ signalling and muscle disease. *European Journal of Biochemistry, 267*(17), 5291-5297.

Martin, P. T. (2003). Role of transcription factors in skeletal muscle and the potential for pharmacological manipulation. *Current Opinion in Pharmacology, 3*(3), 300-308. doi: http://dx.doi.org/10.1016/S1471-4892(03)00050-X

McKinnell, I. W., Ishibashi, J., Le Grand, F., Punch, V. G., Addicks, G. C., Greenblatt, J. F., Dilworth, F. J., & Rudnicki, M. A. (2008). Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. *Nature Cell Biology, 10*(1), 77-84. doi: 10.1038/ncb1671

Mcloon, L. K., Falkenberg, J. H., Dykstra, D., & Iaizzo, P. A. (1998). Doxorubicin chemomyectomy as a treatment for cervical dystonia: histological assessment after direct injection into the sternocleidomastoid muscle. *Muscle and Nerve, 21*(11), 1457-1464.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., & Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes and Development, 10*(10), 1173-1183.

Megeney, L. A., & Rudnicki, M. A. (1995). Determination versus differentiation and the MyoD family of transcription factors. *Biochemistry and Cell Biology, 73*(9-10), 723-732.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., & Gianni, L. (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacological Reviews, 56*(2), 185-229. doi: 10.1124/pr.56.2.6

Molkentin, J. D., Black, B. L., Martin, J. F., & Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell, 83*(7), 1125-1136.

Montarras, D., Lindon, C., Pinset, C., & Domeyne, P. (2000). Cultured myf5 null and myoD null muscle precursor cells display distinct growth defects. *Biology of the Cell, 92*(8-9), 565-572.

Moss, F., & Leblond, C. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *The Anatomical Record, 170*(4), 421-435.

Müller, I., Jenner, A., Bruchelt, G., Niethammer, D., & Halliwell, B. (1997). Effect of concentration on the cytotoxic mechanism of doxorubicin—apoptosis and oxidative DNA damage. *Biochemical and Biophysical Research Communications, 230*(2), 254-257.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H., & Baltimore, D. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell, 58*(3), 537-544.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esuml, E., Li, S., Nonaka, I., & Nabeshima, Y.-i. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature, 364*(6427), 532-535.

Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J., & Konieczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Molecular and Cellular Biology, 15*(5), 2707-2718.

Naya, F. J., & Olson, E. (1999). MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Current Opinion in Cell Biology, 11*(6), 683-688.

Nelson, W. G., & Kastan, M. B. (1994). DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Molecular and Cellular Biology, 14*(3), 1815-1823.

Neville, C. M., Schmidt, M., & Schmidt, J. (1992). Response of myogenic determination factors to cessation and resumption of electrical activity in skeletal muscle: a possible role for myogenin in denervation supersensitivity. *Cellular and Molecular Neurobiology, 12*(6), 511-527.

Olson, E., Arnold, H.-H., Rigby, P., & Wold, B. (1996). Know Your Neighbors: Three Phenotypes in Null Mutants of the Myogenic bHLH Gene MRF4. *Cell, 85*(1), 1-4.

Olson, E. N., Perry, M., & Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Developmental Biology, 172*(1), 2-14. doi: 10.1006/dbio.1995.0002

Pallafacchina, G., François, S., Regnault, B., Czarny, B., Dive, V., Cumano, A., Montarras, D., & Buckingham, M. (2010). An adult tissue-specific stem cell in its niche: A gene profiling analysis of in vivo quiescent and activated muscle satellite cells. *Stem Cell Research, 4*(2), 77-91.

Primeau, A. J., Adhihetty, P. J., & Hood, D. A. (2002). Apoptosis in heart and skeletal muscle. *Canadian Journal of Applied Physiology, 27*(4), 349-395.

Psilander, N., Damsgaard, R., & Pilegaard, H. (2003). Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. *Journal of Applied Physiology (1985), 95*(3), 1038-1044. doi: 10.1152/japplphysiol.00903.2002

Puri, P. L., Bhakta, K., Wood, L. D., Costanzo, A., Zhu, J., & Wang, J. Y. (2002). A myogenic differentiation checkpoint activated by genotoxic stress. *Nature Genetics, 32*(4), 585-593. doi: 10.1038/ng1023

Puri, P. L., Medaglia, S., Cimino, L., Maselli, C., Germani, A., De Marzio, E., Levrero, M., & Balsano, C. (1997). Uncoupling of p21 induction and MyoD activation results in the failure of irreversible cell cycle arrest in doxorubicin-treated myocytes. *Journal of Cellular Biochemistry, 66*(1), 27-36.

Radak, Z., Chung, H. Y., & Goto, S. (2008). Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radical Biology and Medicine, 44*(2), 153-159. doi: 10.1016/j.freeradbiomed.2007.01.029

Radak, Z., Chung, H. Y., Koltai, E., Taylor, A. W., & Goto, S. (2008). Exercise, oxidative stress and hormesis. *Ageing Research Reviews, 7*(1), 34-42. doi: 10.1016/j.arr.2007.04.004

Rapozzi, V., Comelli, M., Mavelli, I., Sentjurc, M., Schara, M., Perissin, L., & Giraldi, T. (1998). Melatonin and oxidative damage in mice liver induced by the prooxidant antitumor drug, adriamycin. *In Vivo (Athens, Greece), 13*(1), 45-50.

Raue, U., Slivka, D., Jemiolo, B., Hollon, C., & Trappe, S. (2006). Myogenic gene expression at rest and after a bout of resistance exercise in young (18–30 yr) and old (80–89 yr) women. *Journal of Applied Physiology, 101*(1), 53-59.

Rawls, A., Valdez, M. R., Zhang, W., Richardson, J., Klein, W. H., & Olson, E. N. (1998). Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. *Development, 125*(13), 2349-2358.

Relaix, F., Rocancourt, D., Mansouri, A., & Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes and Development, 18*(9), 1088-1105. doi: 10.1101/gad.301004

Rudnicki, M., Le Grand, F., McKinnell, I., & Kuang, S. (2008). *The molecular regulation of muscle stem cell function.* Paper presented at the Cold Spring Harbor symposia on quantitative biology.

Rudnicki, M. A., Braun, T., Hinuma, S., & Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell, 71*(3), 383-390.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H.-H., & Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell, 75*(7), 1351-1359.

Ruzinova, M. B., & Benezra, R. (2003). Id proteins in development, cell cycle and cancer. *Trends in Cell Biology, 13*(8), 410-418. doi: http://dx.doi.org/10.1016/S0962-8924(03)00147-8

Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A., & Rudnicki, M. A. (1999). Reduced differentiation potential of primary MyoD−/− myogenic cells derived from adult skeletal muscle. *Journal of Cell Biology, 144*(4), 631-643.

Sabourin, L. A., & Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clinical Genetics, 57*(1), 16-25.

Sakuma, K., Watanabe, K., Sano, M., Uramoto, I., Sakamoto, K., & Totsuka, T. (1999). The adaptive response of MyoD family proteins in overloaded, regenerating and denervated rat muscles. *Biochimica et Biophysica Acta, 1428*(2-3), 284-292.

Schiaffino, S., Bormioli, S. P., & Aloisi, M. (1976). The fate of newly formed satellite cells during compensatory muscle hypertrophy. *Virchows Archive B - Cell Pathology, 21*(2), 113-118.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., & Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell, 102*(6), 777-786.

Simonatto, M., Giordani, L., Marullo, F., Minetti, G. C., Puri, P. L., & Latella, L. (2011). Coordination of cell cycle, DNA repair and muscle gene expression in myoblasts exposed to genotoxic stress. *Cell Cycle, 10*(14), 2355-2363.

Simonatto, M., Marullo, F., Chiacchiera, F., Musaro, A., Wang, J. Y., Latella, L., & Puri, P. L. (2013). DNA damage-activated ABL-MyoD signaling contributes to DNA repair in skeletal myoblasts. *Cell Death and Differentiation, 20*(12), 1664-1674. doi: 10.1038/cdd.2013.118

Singal, P., Li, T., Kumar, D., Danelisen, I., & Iliskovic, N. (2000). Adriamycin-induced heart failure: mechanisms and modulation. *Molecular and Cellular Biochemistry, 207*(1-2), 77-86.

Singal, P. K., & Iliskovic, N. (1998). Doxorubicin-induced cardiomyopathy. *New England Journal of Medicine, 339*(13), 900-905. doi: 10.1056/NEJM199809243391307

Siu, P. M., Donley, D. A., Bryner, R. W., & Alway, S. E. (2003). Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. *Journal of Applied Physiology (1985), 94*(2), 555-560. doi: 10.1152/japplphysiol.00821.2002

Siu, P. M., Donley, D. A., Bryner, R. W., & Alway, S. E. (2004). Myogenin and oxidative enzyme gene expression levels are elevated in rat soleus muscles after endurance training. *Journal of Applied Physiology (1985), 97*(1), 277-285. doi: 10.1152/japplphysiol.00534.2003

Smith, C. K., 2nd, Janney, M. J., & Allen, R. E. (1994). Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *Journal of Cellular Physiology, 159*(2), 379-385. doi: 10.1002/jcp.1041590222

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*(1), 76-85. doi: http://dx.doi.org/10.1016/0003-2697(85)90442-7

Smuder, A. J., Kavazis, A. N., Min, K., & Powers, S. K. (2011a). Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle. *Journal of Applied Physiology (1985), 111*(4), 1190-1198. doi: 10.1152/japplphysiol.00429.2011

Smuder, A. J., Kavazis, A. N., Min, K., & Powers, S. K. (2011b). Exercise protects against doxorubicin-induced oxidative stress and proteolysis in skeletal muscle. *Journal of Applied Physiology (1985), 110*(4), 935-942. doi: 10.1152/japplphysiol.00677.2010

Spicer, D. B., Rhee, J., Cheung, W. L., & Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science, 272*(5267), 1476-1480.

Stathopoulos, G., Malamos, N., Dontas, I., Deliconstantinos, G., Perrea-Kotsareli, D., & Karayannacos, P. (1997). Inhibition of adriamycin cardiotoxicity by 5-fluorouracil: a potential free oxygen radical scavenger. *Anticancer Research, 18*(6A), 4387-4392.

Stone, P., Hardy, J., Broadley, K., Tookman, A. J., Kurowska, A., & A'Hern, R. (1999). Fatigue in advanced cancer: a prospective controlled cross-sectional study. *British Journal of Cancer, 79*(9-10), 1479-1486. doi: 10.1038/sj.bjc.6690236

Sugarbaker, P. H. (2009). Cytoreductive surgery and perioperative intraperitoneal chemotherapy for the treatment of advanced primary and recurrent ovarian cancer. *Current Opinion in Obstetrics and Gynecology, 21*(1), 15-24. doi: 10.1097/GCO.0b013e32831f8f32

Sun, X. H., Copeland, N. G., Jenkins, N. A., & Baltimore, D. (1991). Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Molecular and Cellular Biology, 11*(11), 5603-5611.

Swift, L. P., Rephaeli, A., Nudelman, A., Phillips, D. R., & Cutts, S. M. (2006). Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death. *Cancer Research, 66*(9), 4863-4871. doi: 10.1158/0008-5472.CAN-05-3410

Tajbakhsh, S., Rocancourt, D., Cossu, G., & Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell, 89*(1), 127-138.

Tang, X. D., Santarelli, L. C., Heinemann, S. H., & Hoshi, T. (2004). Metabolic regulation of potassium channels. *Annual Review of Physiology, 66*, 131-159. doi: 10.1146/annurev.physiol.66.041002.142720

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science, 226*(4673), 466-468.

Tisdale, M. J. (2009). Mechanisms of cancer cachexia. *Physiological Reviews, 89*(2), 381-410. doi: 10.1152/physrev.00016.2008

Ustanina, S., Carvajal, J., Rigby, P., & Braun, T. (2007). The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification. *Stem Cells, 25*(8), 2006-2016. doi:10.1634/stemcells.2006-0736

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology, 39*(1), 44-84. doi: 10.1016/j.biocel.2006.07.001

Van der Speeten, K., Stuart, O. A., Mahteme, H., & Sugarbaker, P. H. (2009). A pharmacologic analysis of intraoperative intracavitary cancer chemotherapy with doxorubicin. *Cancer Chemotherapy and Pharmacology, 63*(5), 799-805. doi: 10.1007/s00280-008-0800-0

van Norren, K., van Helvoort, A., Argiles, J. M., van Tuijl, S., Arts, K., Gorselink, M., . . . van der Beek, E. M. (2009). Direct effects of doxorubicin on skeletal muscle contribute to fatigue. *British Journal of Cancer, 100*(2), 311-314. doi: 10.1038/sj.bjc.6604858

Van Remmen, H., Hamilton, M. L., & Richardson, A. (2003). Oxidative damage to DNA and aging. *Exercise and Sport Sciences Reviews, 31*(3), 149-153.

Venuti, J. M., Morris, J. H., Vivian, J. L., Olson, E. N., & Klein, W. H. (1995). Myogenin is required for late but not early aspects of myogenesis during mouse development. *Journal of Cell Biology, 128*(4), 563-576.

Vivian, J. L., Olson, E. N., & Klein, W. H. (2000). Thoracic skeletal defects in myogenin-and MRF4-deficient mice correlate with early defects in myotome and intercostal musculature. *Developmental Biology, 224*(1), 29-41.

Voytik, S. L., Przyborski, M., Badylak, S. F., & Konieczny, S. F. (1993). Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. *Developmental Dynamics, 198*(3), 214-224.

Walters, E. H., Stickland, N. C., & Loughna, P. T. (2000). The expression of the myogenic regulatory factors in denervated and normal muscles of different phenotypes. *Journal of Muscle Research and Cell Motility, 21*(7), 647-653.

Wang, J., & Conboy, I. (2010). Embryonic vs. adult myogenesis: challenging the 'regeneration recapitulates development'paradigm. *Journal of Molecular Cell Biology, 2*(1), 1-4.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell, 75*(7), 1241-1244.

Williamson, D. L., Godard, M. P., Porter, D. A., Costill, D. L., & Trappe, S. W. (2000). Progressive resistance training reduces myosin heavy chain coexpression in single muscle fibers from older men. *Journal of Applied Physiology (1985), 88*(2), 627-633.

Wonders, K. Y., Hydock, D. S., Schneider, C. M., & Hayward, R. (2008). Acute exercise protects against doxorubicin cardiotoxicity. *Integrative Cancer Therapies, 7*(3), 147-154.

Wonders, K. Y., Hydock, D. S., Schneider, C. M., & Hayward, R. (2008). Acute exercise protects against doxorubicin cardiotoxicity. *Integratvie Cancer Therapies, 7*(3), 147-154. doi: 10.1177/1534735408322848

Yang, Y., Creer, A., Jemiolo, B., & Trappe, S. (2005). Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. *Journal of Applied Physiology (1985), 98*(5), 1745-1752. doi: 10.1152/japplphysiol.01185.2004

Yutzey, K. E., Rhodes, S. J., & Konieczny, S. F. (1990). Differential trans activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. *Molecular and Cellular Biology, 10*(8), 3934-3944.

Zammit, P., & Beauchamp, J. (2001). The skeletal muscle satellite cell: stem cell or son of stem cell? *Differentiation, 68*(4-5), 193-204.

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., & Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *Journal of Cell Biology, 166*(3), 347-357. doi: 10.1083/jcb.200312007

Zhou, Z., & Bornemann, A. (2001). MRF4 protein expression in regenerating rat muscle. *Journal of Muscle Research and Cell Motility, 22*(4), 311-316. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11808771

Zhu, Z., & Miller, J. B. (1997). MRF4 can substitute for myogenin during early stages of myogenesis. *Developmental Dynamics, 209*(2), 233-241. doi: 10.1002/(SICI)1097-0177(199706)209:2<233::AID-AJA9>3.0.CO;2-J

Zorzato, F., Salviati, G., Facchinetti, T., & Volpe, P. (1985). Doxorubicin induces calcium release from terminal cisternae of skeletal muscle. A study on isolated sarcoplasmic reticulum and chemically skinned fibers. *Journal of Biological Chemistry, 260*(12), 7349-7355.

Zweigerdt, R., Braun, T., & Arnold, H.-H. (1997). Faithful expression of the Myf-5 gene during mouse myogenesis requires distant control regions: a transgene approach using yeast artificial chromosomes. *Developmental Biology, 192*(1), 172-180.

APPENDIX A

UNIVERSITY OF NORTHERN COLORADO
INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE

UNIVERSITY of NORTHERN COLORADO

IACUC Memorandum

To:	Dr. David Hydock
From:	Laura Martin, Director of Compliance and Operations
CC:	IACUC Files
Date:	8/26/2014
Re:	IACUC Protocol 1407C-DH-R-17 Approval

The UNC IACUC has completed a final review of your protocol "**Nutrition and Exercise in Cancer Treatment-Induced Muscle Dysfunction".** The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 1407C-DH-R-17.

The next annual review will be due before **August 26, 2015**.

Sincerely,

Laura Martin, Director of Compliance and Operations

APPENDIX B

PILOT STUDY

Pilot Study

Tissue samples of SED-SAL (6) and SED-DOX (6) Sprague-Dawley rats, which received injection 5 days prior to sacrifice, were analyzed with Western blot and ECL imaging techniques (Figure 1). Compared to SAL-treated animals, the DOX-treated group exhibited significantly higher levels of Mrf4 and myogenin in the SOL. A trend for decreased Myf5 and $MyoD_1$ expression in the SOL was also observed. The EDL of DOX-treated animals exhibited increased levels of Myf5 and $MyoD_1$ and decreased expression of Mrf4 just above significance.

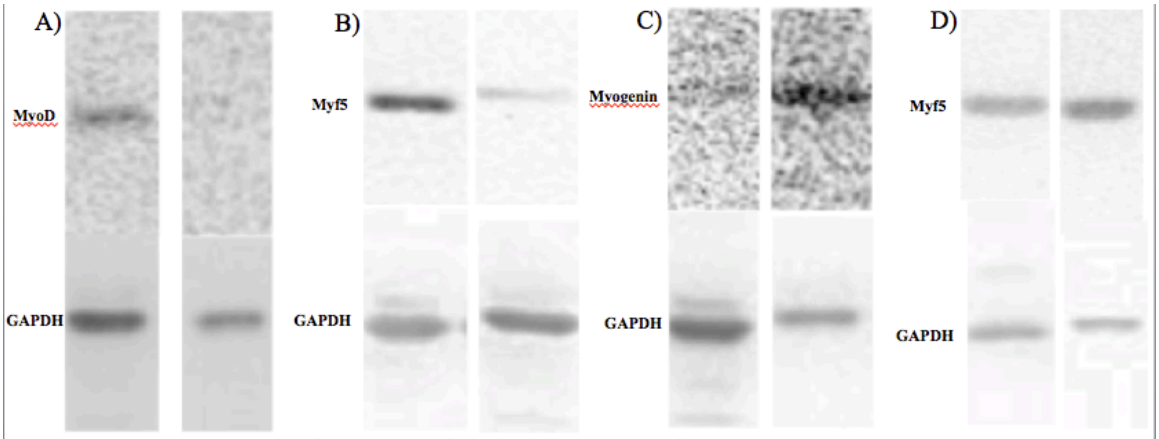

Figure 1. Western blot ECL images of 5-day MRFs above GAPDH (Saline, left; DOX, right)
A) SOL MyoD decreases with DOX; B) SOL Myf5 decreases with DOX; C) SOL myogenin increases with DOX; D) EDL Myf5 increases with DOX

Upon examination of the same hindlimb skeletal muscle tissues of Sprague-Dawley rats 3 days after injections (Figure 2), Myf5 protein levels appear to be much greater in the fast-twitch EDL. When examining Mrf4 levels, bands appear darker in slow, oxidative SOL. These samples were not run with a housekeeping protein to ensure

protein concentrations of samples were uniform. However, protein samples loaded in pilot study were at a concentration of 15 µg. Increased concentrated protein loading will likely elicit higher detection of MRFs. Dedkov and colleagues (2003) effectively identified MyoD and myogenin proteins with Western blot with similar sample concentration (50 µg per lane).

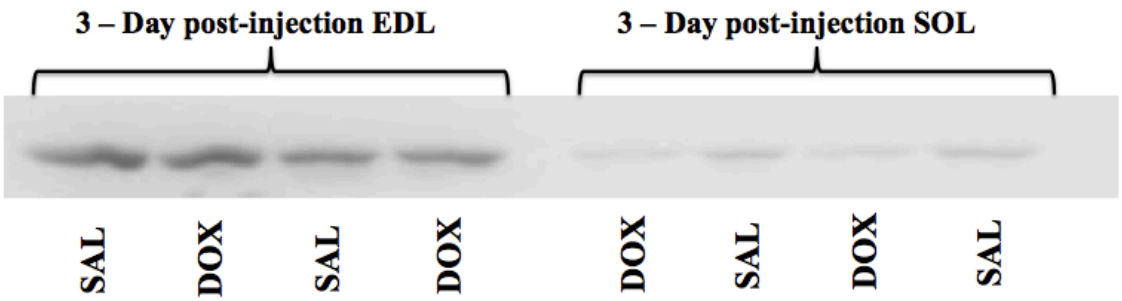

Figure 2. Western blot ECL images of 3-day Myf5 expression in SED rats receiving DOX and SAL
Protein bands appear to be more concentrated in EDL than SOL

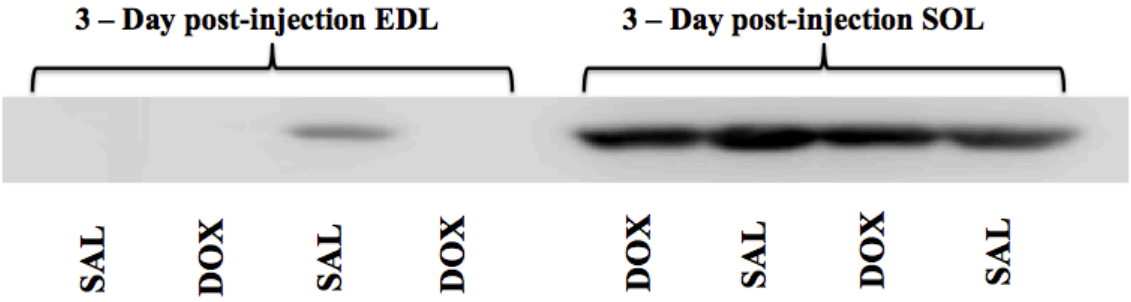

Figure 3. Western blot ECL images of 3-day Mrf4 expression in SED rats receiving DOX and SAL
Protein bands appear to be more concentrated in SOL than EDL

As stated in previous research, a differential expression of MRF proteins exists between fibers types, and may play a role in determining phenotypes. Additionally, DOX appears to alter levels in SED animals. Both resistance and run exercise has been shown

to elevate MRF mRNA levels, but these transcripts return to basal conditions 24 hours following single bouts (Psilander et al., 2003; Yang et al., 2005). Given functional MRF proteins are expressed after mRNA, their presence should be apparent in periods following mRNA expression. However, as shown in denervation studies, mRNA levels may increase without satellite cell activation and no change in muscle mass (Dedkov, Kostrominova, Borisov, & Carlson, 2001; Sakuma et al., 1999; Voytik et al., 1993; Walters, Stickland, & Loughna, 2000). To avoid adaptation to training and return to basal levels of Myf5 and Myod, a 2-week training period will be employed.

Investigating the effects of endurance exercise and DOX treatment on MRF protein expression may provide insight to mechanisms potentially preserving skeletal muscle adaptation and regenerative capacity. This proposed research is a practical study based on supported translational research that can be implemented fairly easily to improve health and well-being in an already at-risk patient population.